FIMBRIATION IN ENTEROBACTERIA

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Thesis presented to The University of Edinburgh for the Degree of Doctor of Philosophy.

September, 1963.

CONTENTS

INTRODUCTION

One of the most striking features emerging from microbiology is that, despite their microscopical dimensions, bacteria show such great diversity with respect not only to their habitat and metabolic activities, but also to their genetic versatility. Until recently, the light microscope, in conjunction with staining techniques, was the only tool with which microbiologists could study microbial structure. Nevertheless, with it they were able to reveal a diversity of structure which manifested itself in the form of different structures at the cell surface - cell wall, capsules (and slime) and bundles of flagella. The requirements of the clinical bacteriologist for the speedy diagnosis of pathogens stimulated research into the serological aspects of these various surface accumulations. When one considers the vast amount of work accumulated in the successful diagnostic systems such as the Kauffmann-White scheme for salmonellas and the Lancefield grouping for streptococci, it becomes apparent that a large amount was known even before the study of these structures was placed on a chemical basis. The impetus of electron microscopy and sectioning techniques allowed the fine structure of cell walls and flagella to be studied, and resulted in the discovery of a farther kind of appendage, thinner and more numerous flagella, called fimbriae. (Houwink and van Iterson, 1950, Duguid, Smith, Dempster and Edmunds, 1955).

These surface structures can be conveniently

classified into two groups (a) those which are appendages radiating from the cell surface - flagella and fimbriae and (b) those which form concentric layers around the protoplast - the cell wall, the micro-capsule and the capsule. In the definition of the layers occurring externally to the protoplast, some confusion exists and the status assigned to particular layers, e.g. the micro-capsule, is not clearly defined. It can be considered as an integral part of the cell wall, or as a separate structure (Salton, 1960). Removal of one or more of these layers or appendages does not result in cell death provided that the external conditions are suitable. Mutations involving the loss of these non-essential structures are known.

When present on a bacterium, each of these structures is capable of performing a specific function important in species survival. The cell wall (i.e. the rigid, muco-peptide layer of the cell wall) is associated with the mechanical rigidity and cell shape, and is resistant to the action of many chemical agents. Research into the micro-capsule layers showed that they are the sites of toxicity of pathogenic species, endowing it with invasive properties. The role of the capsule has been fully discussed elsewhere (Wilkinson, 1958). Suffice it to say that its main roles are protection against bacteriophage and phagocytosis, and prevention of desiccation. The presence of flagella, with few exceptions, bestows on cells the property of loco-

motion enabling them to progress to and explore new environments for colonisation. All these properties, present in an organism either singly or in combination, are obviously important in species survival.

Electron microscopy together with techniques of the biochemist, such as ultra-centrifugation and electrophoresis, were used by the microbiologist, in conjunction with chemical analyses, to reveal the chemical nature of these structures. The discovery of many molecular species such as polysaccharide, protein and lipid, occurring either singly or in complex forms, indicated chemical as well as structural diversity. Differences in the chemical structure existed not only between the different surface structures but also between the same structures from a range of organisms. While this progress was made in the elucidation of the chemical substructure, serological and genetical studies were also revealing variations. Ever since the days of pure culture technique and single-cell lines in bacteriology, it had been obvious that within a bacterial population variation occurred. The variants which arose bred true and so gave rise to types antigenically distinguishable from their progenitors. As well as the gross all-or-none variations which occurred, e.g. complete suppression of flagellation or capsule formation, farther analyses of a genetic and serological nature revealed more subtle variation of the dominant cell antigens.

An excellent example of such modification is seen from the complexity of the microcapsule layer in the gram-negative organisms, in which many antigenic types are found which show different degrees of sharing. The Kauffmann-White scheme used in the diagnosis of salmonellas is based on the serology of these somatic antigens and the diphasic flagellar antigens. The antigenic anatomy of the Salmonella genus is so versatile that approximately 700 species have been serologically distinguished with new serotypes added to the scheme each year. For a long time, the pathways of the microbiological chemist and the serologist went their individual ways, and only recently has contact been made. More frequent collaboration in the two fields would be desirable so that the evaluation of antigenic variations could be understood chemically. The detailed chemistry of these structures is, as yet, understood in but a few cases (see Davies,1960), but it is to be hoped that, where possible, future genetical evidence will be equated with chemical investigation.

While the presence of extraprotoplastic hetero- and homopolymers is important in species survival simply by the functions they fulfil, the characteristic ability of a bacterium to modify these antigens is equally important. For example, in the case of pathogenic organisms, it is well known that antibodies are produced by the host defence mechanisms to the cell antigens

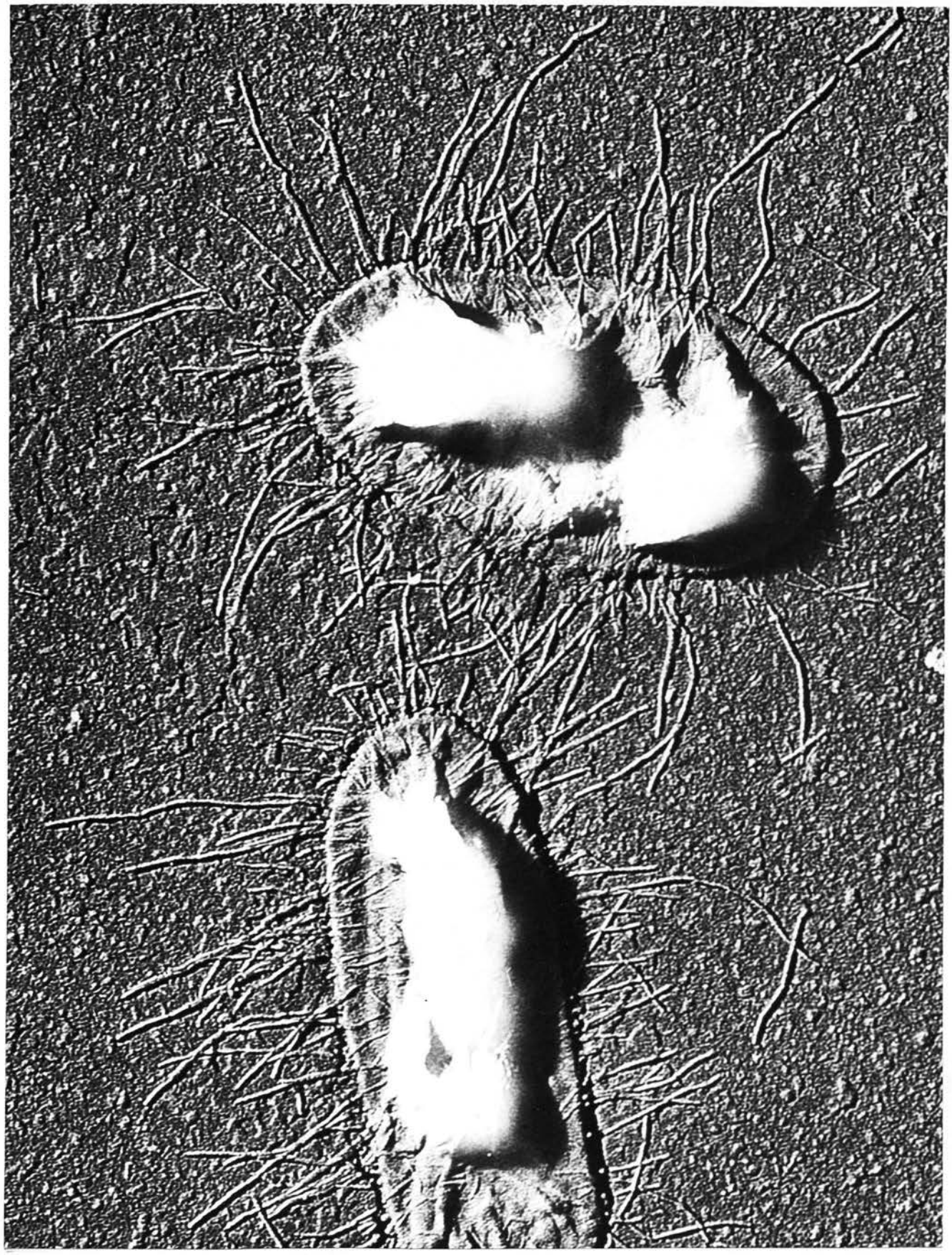


Fig.1. Escherichia coli, strain 23, from aerobic static broth culture (48 hr. at 37°), as used in the investigations on the chemical nature of fimbriae, reported in Section II (mag. x 96,000)

situated externally to the innermost cell wall layer. It is clear that bacteria with a modified antigenic structure will be well suited to adapt and survive. In this way, increased survival efficiency becomes synonymous with increased structural modification, and gives a partial explanation of the complexity of the bacterial surface. An additional hazard to both pathogenic and non-pathogenic organisms will be attack by colicins and bacteriophage, each of which has specific sites for adsorption at the cell surface. Changes in these sites, i.e. variation in the surface antigens, will be associated with the origin of resistant variants.

As mentioned earlier, the advances made in electron microscopic technique led to the discovery of fimbriae. Since the first objective studies were made on fimbriae (Smith, 1954, Brinton, Buzzell and Lauffer, 1954, Duguid et al, 1955) many functions have been proposed for them, none of which was unequivocally upheld. Such dominant antigens as fimbriae are obviously capable of exerting strong effects on the bacterial surface, especially since it has been shown that a number of inter-changing variants exists. It is surprising, therefore, that these appendages have been so neglected. At the outset of this investigation, little was known of their chemical structure, their relationship to other surface accumulations or their genetic control. With these deficiencies in mind, it was proposed to investigate them

from three different aspects (1) their genetic control (2) their function and (3) their chemical structure. Although little existed in the way of published data specific to the fimbriation studies, it seemed that many of the methods and approaches successfully applied to other cell antigens, particularly flagella, would prove adaptable.

In reviewing the literature, it is expedient to include summarily short sections on the cell wall, micro-capsule and the capsule. The literature on these three surface layers is too vast to be coherently reviewed here, but excellent reviews are available (Wilkinson, 1958, Davies, 1960, Staub, 1960, Westphal, 1960, Salton 1960 and 1961, Work, 1961, Cummins, 1962 and Strominger, 1962). Recently the number of recognised mechanisms of genetic transfer available to gram-negative organisms has increased so vastly, that it seemed necessary to give a short review of the known methods of genetic recombination. It became obvious during the course of this research that some of the unusual methods had to be considered to explain certain phenomena. Particular reference is made to the analysis of genetic regions by transduction, since this was the method used by the author in the investigation of the control of fimbriation.

CHAPTER I

The bacterial surface.

1. Surface layers.

a. The cell wall (and microcapsule).

Quantitative analyses of the cell walls of gram-positive and gram-negative organisms revealed the presence of a "basal" structure of which the constituents are acetyl-glucosamine, glucosamine, acetylmuramic acid, alanine, glutamic acid and either lysine or diaminopimelic acid. This portion of the cell wall, referred to as the muco-peptide layer, is responsible for cell rigidity. Cell walls of both gram-positive and gram-negative organisms contain additional components (referred to as a "special" structure, Salton, 1960, Strominger, 1962). In gram-positive organisms, this additional structure is usually small e.g. in Staphylococcus aureus the only additional constituents are glycine and the teichoic acids, although more complex "special" structures are known e.g. in corynebacteria and streptococci. These additional components give to an organism its characteristic sero-logical behaviour. In gram-negative organisms, the additional layer is an extensive protein-polysaccharide-lipid complex constituting up to 80% of the cell wall dry weight. This outer, pliable layer of the cell wall contains lipopolysaccharides (endotoxins or micro-capsules). The two layers are best separated by phenol extraction.

The lipopolysaccharides can be separated to give lipids, which are the active groups determining antigenicity. Although a great deal of work has been done on these lipopolysaccharides (see Davies, 1960, Staub, 1960, and Westphal, 1960) their detailed chemical structure is known in only a few cases. Classification of the polysaccharides by their sugar composition (i.e. chemotype) gives a picture which confirms the classical serology of the Kauffmann-White scheme.

b. The capsule.

This was the first of the surface layers to be removed and characterised chemically. It is a polymer of a single molecular species, either polysaccharide or protein. The ability to synthesise extracellular polysaccharide into a capsule can be lost. When this occurs, non-adherent slime is produced, which is chemically like that of the parent capsular material (see Wilkinson, 1958, Salton, 1960).

II. Appendages.

a. Flagella.

These are sinuous filaments, arranged polarly or peritrichously on the bacterial surface, and found in both gram-positive and gram-negative organisms. Removal of the cell walls of lysozyme-sensitive bacteria by this enzyme yields protoplasts which are flagellate and non-motile. This, and the supporting electron microscopical evidence of granules at the site of origin of flagella,

indicates that flagella arise from within the cytoplasmic membrane. Although the function of the basal granule is not known, it might be the site of energy generation for locomotor function.

Flagella are simple structures varying in thickness from 12 - 19 mμ, according to the species examined. The only reports of complexity of structure in flagella came from early workers. The presence of a fragmentary trypsin-sensitive sheath surrounding a trypsin-resistant central flagellar filament was claimed in Bacillus brevis (De Robertis and Franchi, 1951). Their electron micrographs were prepared after the bacteria had been treated with trypsin for 11 hr. A thick polar flagellum was also claimed in Vibrio metchnikovii by van Iterson (1953). In both cases, failure to examine control preparations and electron micrographs of low quality make their conclusions seem unlikely. More likely these sheaths were contaminating material from other surface structures which had aggregated on the flagella. Neither report has been verified by recent workers. Examination of untreated bacterial flagella by electron microscopy gives no evidence of an organised structure (Kerridge, Horne and Glauert, 1962). Surface irregularities on the flagella have been interpreted by other authors as evidence of helical formation (Labaw and Mosley, 1954). It would seem that most of these early reports of complexity in the structure have resulted from an over enthusiastic interpretation of

the available evidence.

Much of the chemistry of the bacterial flagella has been done by Weibull (1948, 1949, 1951 and 1953) and Koffler and his associates (1956, 1957). Flagella from six different species have been purified by differential centrifugation and ammonium sulphate precipitation. Purified flagella, containing negligible amounts of phosphorus, lipid, carbohydrate and nucleic acid, gave a nitrogen content of 15.7 - 16.1% of the dry weight, which indicated that flagella contained at least 99% protein (which is called flagellin). The qualitative difference in the amino acids of the different flagellins is slight (see Kerridge, 1961). Flagellin is an incomplete protein lacking tryptophane, histidine and hydroxyproline of the common amino acids. Studies on the regeneration of bacterial flagella after their removal by high-speed blending showed that in the absence of these amino acids and in the absence of general protein synthesis, bacterial flagella were resynthesised in the absence of growth. They could also be resynthesised in the absence of methionine, an amino acid occurring in only small amounts in the flagellin of Salmonella typhimurium (Kerridge,1959a). The known wide range of flagellar antigens, especially in Salmonella, is probably due to differences in the amino acid sequence of the different flagellins.

Flagellins are antigenically multivalent, inducing different H antibodies to different parts of the protein

molecule. This was suspected previously from the work of Nakaya, Uchida and Fukumi (1952), who performed agglutination tests on the detached flagella of S.enteritidis which has the flagellar components g and m. All flagella were precipitated by either anti-g or anti-m serum, showing that the two determinants resided on a single flagellum. However, Gard, Heller and Weibull (1955) revealed by gel-diffusion tests two flagellar H antigens in Proteus flagella. The major antigen was identical with the homogeneous protein of molecular weight of 41,000, retaining its full antigenicity even after three hours heating. The minor component, although heat labile, was also regarded as flagellar. Their purified preparations always stimulated O-antibody production, and it would seem that this second minor component might be associated with contaminating O-antigen material. It is also known that immunodiffusion tests can give a number of bands of precipitation in excess of the number of antigen-antibody systems present, when subjected to sudden variations in temperature or unbalanced reactants ratio (see Crowle, 1960). These possibilities must be considered here. Unfortunately, the literature on gel-diffusion tests with flagellins is sparse, and the discrepancy cannot be resolved.

The flagella of most strains of Salmonella assume two distinct phases which alternate rapidly and reversibly.

This phase variation, unlike fimbrial phase variation, is not environmentally controlled. Change from one phase to the other can be induced by growth in the presence of specific antiserum to the individual phases. Phase I (specific) contains the more specific antigens, symbolised by the terms a, i, g, m ... and phase 2 (group) antigens the less specific, symbolised by 1, 2 ... e, n, x, e.g. Salmonella typhimurium (i: 1,2). Transduction studies by Lederberg and Edwards (1953) and Kauffman(1953) showed that the two phases were controlled by distinct genes H_1 and H_2. Regardless of whether the recipient strain was monophasic or diphasic, the antigens of one phase were replaced by antigens of the homologous phase only, and the component factors of the flagellar antigens (e.g. 1, 2, 3 or e, n, x) did not separate during transduction. This was farther evidence that the component flagellar factors were resident on a single molecule of flagellin,and not represented each by its own specific flagellin. These transduction studies confirmed the classification by the Kauffmann-White scheme, and showed that the different antigens in a phase represented a series of alleles. The new combinations of antigenic structure achieved were often difficult to classify by the Kauffmann-White scheme and tend to be excluded from this, unfairly so, since unnatural types arising spontaneously have been reported (Bruner, 1953).

Although substitution during transduction usually

occurs between antigens of homologous phase, Lederberg (1961) showed that S.paratyphi B CDC 157 had the genotype $H_1^{b}H_1^{1,2}H_2^{?}$, while typical strains of this serotype are $H_1^{b}H_2^{1,2}$. Transduction to CDC 157 had shown the phase and homologies of the 1, 2 antigen. Its progeny (by transduction) revealed unusual serotypes such as 1,2:1,5 and 1,2:e,n,x. This exception was best explained by a duplication of the H_1 locus (controlling phase 1 antigens) by unequal recombination. Such exceptional findings are always interesting, but more valuable if verified. Duplication of H_1 locus was shown also by Iino (1959b and 1961). From a cross between S.typhimurium (i:1,2) and S.abony (b:e,n,x), he isolated an abnormal (i:b) type which expressed alternately the phase 1 antigens of the two parents. In each of these cases, the control of the normal phase 2 flagellin has been blocked and replaced by abnormal synthesis of the two phase 1 flagellins. It is probably with such unusual types as these that the problem of phase variation will be solved.

Monophasic strains forming only one species of flagellar antigens occur naturally, e.g. S.abortus-equi (-:e,n,x) and S.enteritidis (g,m:-). Although not expressed, the overt antigens could be sought by transduction methods, using the monophasic strain as both donor and recipient. For example, latent phase 1 antigen a could be transduced to and from S.abortus-equi ((a):e,n,x) showing that this strain was capable of forming a-type

flagellin, although this synthesis was usually not expressed. S.paratyphi B (b:1,2) is normally a diphasic strain, but Lederberg and Edwards (1953) showed the existence of a stable monophasic form incapable of forming H_2 flagella (b:-). Use of this strain as a recipient showed its ability to form H_2 flagella, although it did not, when used as donor, evoke the incorporation of the H_2 locus in recipient strains. Thirdly, Lederberg and Edwards (1953) were unable to transduce phase 2 antigens in S.typhi (d:-) with a range of strains showing a wide H_2 antigenic spectrum. Since it also failed to donate the H_2 locus when used as a donor, it was concluded that this monophasic strain never contained the H_2 gene. Iino(1957) found stable monophasic variants of S.typhimurium in which the phase 1 flagella were not found, and presented evidence of genes Ah_1 and Ah_2, closely linked to the H_1 and H_2 genes, but not part of them. These genes apparently inhibited the synthesis of flagella of the phase not expressed, and it was unfortunate that this analysis was not extended. One feature which emerges from the analysis of the genetic determinants of flagellation is that, although there is evidence of specific genes controlling definite aspects of flagellation, these are so clustered around the genes H_1 and H_2 that analysis, even by transduction, is difficult.

The H_1 and H_2 genes are good examples of structural genes, apparently controlling the antigenicity by controlling the amino acid sequence of the different

flagellins they produce. It would seem that the most likely mutation occurring in the H_1 and H_2 genes would be one in which a single amino acid was altered. Such a mutation would result in the synthesis of a flagellin almost the same as the parent flagellin expressing, perhaps, a minor change in antigenicity. Investigation along these lines were initiated by Iino (1959a) with the phase 1 antigens of the "g" ... series. Although evidence had shown that these component factors of a particular phase antigen were not segregated during transduction (Lederberg and Edwards, 1953) serological studies revealed that the factors separated and variant forms were produced. Iino's work indicated that there were 8 major components - f,g,m, p,q,s,t,u - each of which mutated independently. All combinations of two factors randomly chose from g,m,f,p, and t are known. The u factor was found with g,m,p and t, but not s. The f factor was detected in combination with g or both g and t, and the q factor with g or with g and m. From this data, he subdivided the H_1 locus into five antigenically distinct sections. This demonstration of antigenically specific areas on the flagellin molecule confirmed the earlier work of Nakaya et al (1952). Unfortunately, this work was not correlated with differences in the chemical structure of the different antigens.

Another example of the detailed analysis of the H_1 locus is that by Joys and Stocker (1963), who analysed

the *i* flagellin of *S.typhimurium.* By inoculating this organism into semisolid plates containing excess phase 2 (1,2) serum and just-immobilising concentrations of phase 1(i) serum, they isolated a series of mutants in the *i* locus differing slightly from the parent. This change in the H_1^i gene had, therefore, resulted in changes in serological character, but not in a loss of locomotor ability. Reciprocal transduction among the mutants allowed them to map the mutations involved, but until the amino acid sequence of flagellins is known, it is not possible to discuss the mutations in terms of changes in the amino acid sequence along the polypeptide chain. This work was correlated with that of McDonough (1962) who showed tryptic peptide maps from the flagellins of some of the mutants were different from that of the parent wild-type *i* flagellin.

Thus, the genetic analysis of Salmonella flagellation is now approaching a stage where changes in antigenic character of flagellins can be correlated with changes in their chemical structure. However, although the literature on the control of flagellar antigenicity is wide, this is not the only aspect of flagellation which has received attention. The earliest workers in this field recognised that other aspects were involved in the determination of Salmonella flagella. The actual presence or absence of flagellar synthesis was known to be controlled by a locus *fla+* close to the structural genes H_1 and H_2. Evidence of allelism among strains of *Salmonella typhimurium* in which there was complete suppression of flagella

synthesis in both phases emerged from reciprocal transduction studies among nine fla- mutants. The presence of six interacting groups suggested at least an equal number of loci controlling flagellation, mutation at any one of which resulted in an absence of flagella, Stocker et al (1953). Iino (1958b) showed that four of twelve fla- S.typhimurium strains produced functional flagella in reciprocal transductions with any other strain. Six of the twelve strains, however, when crossed, gave only partial restoration to the wild-type functional flagella. His results enabled him to map the mutations involved in the fla locus, which he found to be close to H_1. This finding of close linkage of fla and H_1 was confirmed by Smith and Stocker (1962) using another technique of genetic transfer, namely, recombination by colicinogenic factors. Exceptions to this close linkage of fla and H_1 have been found with other non-flagellated mutants, mutated, for example, at a locus fla 29 between gal and try B, i.e. to the left of H_1.fla_1 on the single circular chromosome, (Smith and Stocker, 1962).

Yet another aspect of control not concerned with antigenicity or formation of flagella emerged. Bacteria are known which have flagella, but are non-motile. A gene mot controlling locomotor function was recognised as early as 1953 (Stocker et al) who suggested that at least two loci controlled the function of flagella, which was later increased to four non-identical loci by transductions between four paralysed, flagellated strains (Iino, 1958b).

The morphology and serological characters of the paralysed flagella are quite normal as tested by cross-absorption tests, and it is not known why the flagella are non-functional. It is conceivable that the gene mot controls the activities of the basal granule, and that these mutations represent defects in this structure.

Phenylalanine is a constituent amino acid of the flagellin of all species examined. Elegant experiments by Kerridge (1959b) demonstrated that when bacteria, deflagellated by blending, were allowed to regenerate their flagella inthe presence of the amino acid analogue, p-fluorophenylalanine, this analogue was incorporated in the flagellin synthesised. This resulted in flagella which were morphologically changed to a form showing smaller wavelength, and incapable of translational motility. These are referred to as "curly" flagella. "Curly" flagella occur naturally in some strains in the absence of analogues. These strains too perform only rotational movements and fail to swarm on semisolid media.
Iino (1958a, 1962) showed that Salmonella typhimurium SW577 has normal flagella in phase 2, but "curly" flagella in phase 1. These flagella are antigenically similar to the normal flagella as judged by cross-absorption tests with isolated flagella of both kinds. Transduction from a normally flagellated strain to a "curly" phase 1 recipient strain gave normal flagella types, showing donor and recipient antigens in phases 1 and 2 respectively. This

located the "curly" gene close to the H_1 determinant, and gave a further indication of the complexity in this H_1 region.

Seven mutants of S. abortus-equi were described with "curly" flagella in either phase 1 or phase 2 (Iino, 1962). Transduction analyses confirmed the close relation of the "curly" determinant of phase 1 and the H_1 gene, and showed that the phase 2 "curly" determinant was linked to the H_2 antigen-controlling gene. We see, therefore, that the functions controlled by the genes curly, mot and H_1 - morphology, morphogenesis and antigenicity - are quite distinct, and that mutations can result in any one of these genes without affecting the other two (except that "curly" mutants have a restricted type of motility).

Variation in the chemical structure of certain flagellins was found to be caused by an unusual amino acid, ε-N-methyl lysine (Ambler and Rees, 1959). The presence of such an amino acid in proteins was thought to be controlled by a specific gene other than the structural gene which controls the amino acid sequence of a protein. When present, ε-N-methyl lysine occurs in the flagellins of both phases, e.g. S.typhimurium and S.derby (Stocker, McDonough and Ambler, 1961). Analysis showed that its presence in flagellins was controlled by a gene nml closely linked to H_1 and fla. Replacement of the phase 2 determinant by transduction did not affect the N-methyl lysine character of the strain and the presence of this amino acid in phase 2 flagellins was controlled by a gene

not close to the H_2 locus. This argued that the genes H_1 and H_2 controlled the amino acid sequence (including lysine) in the synthesis of flagellin. The methylation of lysine, occurring after incorporation of lysine into the flagellin, is specified by an enzyme whose production is controlled by the gene nml.

Finally in this section, we consider the variation between phases 1 and 2 which occurs at high rates, 10^{-4} and 10^{-5}, with the variation from 2 to 1 being slightly faster (Stocker, 1949). This ability to rapidly synthesise an appendage of different antigenicity will be of most advantage to pathogenic organisms attempting to counteract the antibodies produced by a host defence mechanism. Lederberg and Iino (1956) explained phase variation in a limited number of strains by the "state" of the dominant H_2 locus, which could be either active, in which the H_2 antigens would be expressed, or inactive, in which the H_1 antigens, no longer suppressed, would become phenotypically expressed. The H_1 activity was, therefore, H_2 - state dependent and, in this way, monophasic types might be explained by an absence of the H_2 active state. The control of this phase variation was not, and is not, known, and the only further data which appeared was a short communication by Iino (1958) in which he claimed that a gene Vh_2 close to H_2 controlled the variability of the Phase 2 antigens. This locus was not defined in detail, and no evidence was presented then or later of a similar locus

controlling phase 1 antigen stability. It is unfortunate that this very important aspect of flagellation in Salmonella has not received more attention since then.

Since that time, important new discoveries have been made whose involvement in this system might be considered, even in the absence of experimental evidence. The class of genetic determinants called episomes have received a great deal of attention in the last few years (see Jacob, Schaeffer and Wollman, 1960). Summarily, episomes are dispensable cell components which can be lost irreversibly without damage to the cell. They express on a bacterium three different phenotypes depending on whether they are absent, integrated to the bacterial chromosome or existing cytoplasmically. They are elements which can control certain cell processes and bestow advantages on a bacterium which possesses them. Any function is more likely to be episomically controlled if it is a non-essential feature and an alternately expressed one. Flagellar phase variation would seem to be an obvious function which might prove to be under episomic control.

It might be that the structural genes H_1 and H_2 are incapable alone of synthesising flagella, doing so only in the presence of an additional determinant or episome. It is possible that each structural gene is controlled by its own episome, the synthesis of the particular flagellin being dependent on either the integrated or autonomous state of its episome. If, for the sake of argument, the episome in its integrated state

co-ordinated with the structural gene to produce the synthesis of flagellin, then this process might be inhibited by its transition to a cytoplasmic state. Irreversible loss of the episome would then account for the origin of monophasic types. This model would explain the control of the synthesis of the two types of flagellin, but would fail to explain the co-ordination of phase variation. Alternatively, still arguing the requirement for an episome for full expression of the structural gene, it might be that a single episome alternates between an integrated state on one structural gene and then another at a very rapid rate. The dominance of the states by the H_2 active locus could then be explained by a preferential site of attachment on the H_2 structural gene. Its attachment to a particular gene would stimulate the synthesis of flagella of the correct phase. Monophasic strains could then be explained by the permanent attachment of the episome to one structural gene, although such a simple picture is insufficient to explain the different monophasic types.

In bacteria, in addition to structural genes, control genes are found which are of two types. The first is a regulator gene determining the production of a specific repressor substance which controls the expression of a structural gene by preventing excess production of a molecular species in a cell. The second is an operator (switch) gene which controls the function of one or several

genes adjacent on a chromosome. The operator genes (which usually are found at the end of a segment in which there are several genes controlling enzymes in the same metabolic pathway) are the supposed sites of repressor action (Jacob and Monod, 1961). Although operator genes have not been discovered controlling the H_1 and H_2 structural genes, it is not difficult to envisage such a control mechanism. The synthesis of flagella of a phase could be inhibited by the production of a repressor, specific for the operator of that particular phase. The rapid induction and repression of the two phases would need to be co-ordinated, and this switch might come about by the alternate states of the common episome. Its presence, as well as being necessary for the synthesis of flagella in co-ordination with the structural gene, might also stimulate the production of a repressor substance inactivating the operator of the other phase. In the absence of any experimental data, and the difficulty of adapting any theory to include the many exceptional cases we have observed, it is wiser to theorise no further.

b. Fimbriae.

Description.

Filamentous appendages other than flagella are found at the cell surface of a number of gram-negative organisms. These are known as fimbriae (Duguid et al,1955) or, less commonly, pili (Brinton, 1959). Their morphology, which can be seen only by electron microscopy, is quite

unlike that of flagella. Fimbriae are peritrichously arranged filaments, several hundred per cell, extending in length up to 4μ. Their diameter varies according to the type examined, but is never more than 7mμ. The absence of wave form is suggestive of a structure more rigid than that of flagella.

Discovery, early reports and inferred relationship to other surface structures.

Among the earliest reports was that by Anderson (1949) who suggested that these "tenuous threads" might be artefacts resulting from the drying of capsular material during electron microscopical preparation. However, Duguid et al (1955) found no correlation between presence of fimbriae and capacity for capsule formation in Escherichia coli strains grown on excess sugar media to stimulate capsule and slime formation. Their electron micrographs clearly differentiate between the regular structure of the filaments and the hazy regions caused by capsule or slime, and fimbriae and capsular material are shown on the one organism. This finding was confirmed by the discovery of fimbriae in the permanently non-capsulated Shigella flexneri (Duguid and Gillies, 1957). Houwink and van Iterson (1950) were the first to give a detailed description of fimbriae. Although most early workers assumed them to be variant forms of flagella and referred to them as "pseudoflagella", (Noda and Wyckoff, 1952, Smith, 1954), Houwink and van Iterson suggested that these organs might perform other functions.

Species occurrence.

Houwink and van Iterson had shown the presence of fimbriae in Escherichia coli, Photobacterium splendidum and Pseudomonas pyocyanea. Duguid and Gillies (1958) surveyed a large number of bacterial species of different genera and found fimbriae in Shigella flexneri, Chromobacterium prodigiosum and in most strains of Escherichia, Salmonella, Klebsiella and Proteus. They are also found in Cloaca cloacae (Constable, 1956) and the Providence group (Wright, personal communication). Whether this restricted species distribution is correlated with physiological or functional factors is difficult to judge because of a lack of information on either of these aspects. Their absence from gram-positive organisms is a finding not yet satisfactorily explained. The cell wall of gram-positive organisms is known to have a less complex "special" structure than that of gram-negative organisms. The idea occurred that fimbriae might be another manifestation of the "special" structure, and such theories are still being advanced (Ogura, 1963). However, this plausible theory was discarded when it emerged during the course of this work that the two structures were chemically unalike.

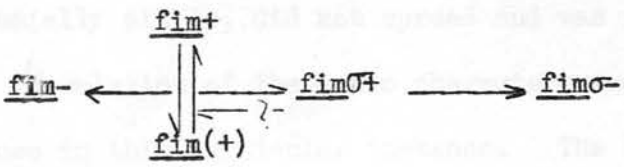
Types of fimbriation.

Reports from different groups of workers agreed that within one species two different kinds of cell were present (a) those which were fimbriate under suitable environmental conditions, designated fim+ and (b) those

which were always non-fimbriate regardless of the cultural conditions, designated fim-. Both Brinton et al (1954) and Duguid et al (1955) showed that within a strain of group (a) a phase variation occurred freely between a non-fimbriate and a fimbriate phase. Each phase changed spontaneously to the other at the high rate of 10^{-3} per bacterium per cell generation (Brinton et al, 1954). The fimbriate variant of a fim+ strain could be selected by either aerobic or microaerophilic static-broth subculture: anaerobic or shaken broths, or subculture on nutrient agar led to the selection of the non-fimbriate variant. (Duguid et al,1955, Duguid and Gillies, 1957). The two types were designated fim + and fim(+), respectively, the latter signifying that the strain is genotypically fimbriate but in a non-fimbriate phase.

Fim+, fim(+) and fim- strains have not shown distinguishing colonial morphology in the many strains examined. However, Brinton discovered that on solid media, fimbriate strains were smaller and smoother than the non-fimbriate (1957). It was later shown that certain permanently fimbriate (P+) strains of E.coli B gave rise to morphologically distinct colonies from which permanently non-fimbriate (P-) forms were mutated at the high rate of 10^{-4} per cell per generation (Brinton, 1959). This presented a completely new aspect of fimbrial phase variation not hitherto recognised. These two extreme views were resolved by Maccacaro, Colombo and di Nardo (1959) who found that

fim+ fimbriate strains of E.coli K 12, when plated on nutrient agar after a long series of subcultures in fluid medium, gave rise to a few, small, compact colonies composed wholly of fimbriate cells. This type which they designated fim σ+, remained fimbriate when subjected to the environmental conditions controlling the fim+ ⇌ fim (+) variation. Maccacaro and Hayes (1961a), therefore, recognised five types of fimbriate and non-fimbriate cells in E. coli, and understood the inter-relationship of types as follows:-



The fimσ+ and fimσ- types apparently correspond to the P+ and P- types of Brinton (1959).

This diversity of types, if present in other species, has not been recognised. In Salmonella and Shigella species, most strains examined seem to be subject to environmentally controlled phase variation, although exceptions are known. Cloaca cloacae species seem to be fimbriate under all environmental conditions (Constable, 1956). Certainly, there is no evidence in these examples of a change in colonial morphology accompanying these types of fimbriation. However, Duguid (personal communication) showed a difference in colonial morphology in a single strain of many thousands examined. Salmonella typhimurium Sa 519 is a non-fimbriate strain which forms a large, smooth colony and occasionally mutates to a small colony form which is fimbriate. Genetical

results reported in this investigation indicate that this change in morphology is not specifically related to fimbriation. Another association between fimbriation and colonial morphology was revealed by Shedden (1962) with Proteus hauseri strains which, on MacConkey's agar, give rise to three phases identifiable by their colonial appearance. When pure phase lines were passed through broth subcultures, it was found that the A and C forms, both spreaders, were fimbriate. The B phase, which was colonially stable, did not spread and was non-fimbriate. The correlation of these two characters was probably one of chance in this particular instance. The author apparently did not consider it worthy of discussion and makes no mention of his finding as significant

Electrophoretic mobility associated with fimbriae.

Brinton and his coworkers (1954, 1959) demonstrated that the electrophoretic mobility of free fimbriae was the same as that of the fimbriate bacteria over a wide range of pH,6 - 11, and ionic strength, 0.003-0.I. This showed that mobility of fimbriate cells was due to the presence of fimbriae. Maccacaro and Turri (1959a) used a microelectrophoretic technique to analyse the ratio of fimbriate and non-fimbriate cells in any culture. They concluded that "since there was an evident bimodality the majority of cells were either completely fimbriate or non-fimbriate". Heating the fimbriate cells increased their electrophoretic mobility, presumably by detaching the fimbriae. This observation is

of considerable relevance to the problem of bacterial growth and cell division. Duguid and Wilkinson (1961) also showed a bimodal distribution directly associated with fimbriae. Electron microscopical observations on exponential phase cells showed that cells were either fully fimbriate or completely non-fimbriate with few exceptions, (e.g. they showed occasional dividing bacteria in which one cell was fimbriate and the other non-fimbriate). The even distribution of fimbriae in most dividing bacteria argues in favour of the theory that cell division is by binary fission, and that the fimbriae present are shared between the two daughter cells. Observations on the distribution of flagella in dividing salmonellas are consistent with this theory (Quadling and Stocker, 1962). These findings present strong evidence against the unipolar ("budding") method of growth proposed by certain workers (see Bisset and Pease, 1957, Bisset and Hale, 1960).

Brinton (1959) showed that the free boundary electrophoresis pattern of a crude fimbrial preparation consisted of two distinct peaks. Samples of each homogeneous peak revealed the leading peak as fimbriae, the trailing one as spheroidal material. Homogeneity of behaviour has been considered as a satisfactory criterion of purity for fimbrial preparations. That this, in the absence of other criteria, is insufficient to establish the purity of a fimbrial preparation is now recognised (Brinton, personal communication). The inadequacy can also be seen from the fact

that fimbriate bacteria and free fimbriae have almost identical mobility patterns (Brinton *et al*, 1954).

Possible functions of fimbriae.

1. Adhesive functions.

Not only were Houwink and van Iterson the first to describe fimbrial morphology, they were also the first to suggest that specific functions might be fulfilled by these organelles. From their electron micrographs, which showed that fimbriae attached themselves to the supporting collodion membrane, they suggested that fimbriae had adhesive properties. Their hypothesis that fimbriae might act as a food-gathering structure capable of utilising the organic substances adsorbed on solid substrata is an attracive one, which has not been verified or denied. Duguid *et al* (1955) confirmed that fimbriae had adhesive properties by correlating their presence in *E.coli* strains with the ability to adhere to and agglutinate red blood cells of various species. They concluded that the agglutination was not enzymatic since it occurred over a wide range of temperature and pH, and was not inhibited by a number of enzyme-inactivating agents. This fimbrial haemagglutination is not a specific reaction because they also agglutinate various plant and animal cells, e.g. leucocytes, epithelial cells, *Candida albicans* and *Penicillium notatum* spores (Duguid *et al*, 1955).

2. Invasive functions.

The significance of these findings in relation to an organism in its natural habitat is not clear. The ability

to adhere to red blood cells can hardly be advanced as valuable to an organism, but the broad spectrum of substrates to which they adhere is interesting. Duguid and Gillies (1957), investigating this phenomenon in fimbriate dysentery bacilli, showed that adhesion to epithelial cells was a rapid reaction. While this might be advantageous in the nutrition of both pathogens and commensals, by enabling them to retain contact with a ready source of nutrients, its importance in pathogenicity was doubtful. Correlation between the presence of fimbriae and invasiveness and pathogenicity is not obvious, when one realises that some of the most successful pathogens are never fimbriate, e.g. Shigella dysenteriae and Salmonella paratyphi A. The finding of Duguid et al (1955) that maximal adhesive activity (with reference to fimbrial haemagglutination, admittedly) occurred at temperatures well below the normal body temperature of the hosts of pathogenic enterobacteria agrees with the hypothesis that fimbriation plays no role in the invasive stages of a pathogen.

Apparently contradictory results were obtained by Campbell (1961) who made a comparison of invasiveness and pathogenicity in fimbriate and non-fimbriate strains of Salmonella typhimurium. He concluded that fimbriate strains showed greater infectivity and virulence than non-fimbriate strains when injected orally into mice. This difference was not present on intraperitoneal injection. Fimbriation conferred no such advantages to a strain in the oral infection of chicks. It might be argued that this reflects that the

natural host of Salmonella typhimurium is rodents and not chicks. However, Duguid (personal communication) considers that this strain has undergone a host transition from rodents to fowl. Several criticisms can be made of Campbell's findings (I) the range of ID_{50} values quoted as significant for compared fimbriate and non-fimbriate strains overlaps considerably in many examples, (II) unrecognised strain characters, such as virulence factor, might have been responsible for the apparent difference in naturally isolated fimbriate and non-fimbriate strains, (III) fim+ and fim (+) cells would not seem ideal for comparison since fimbriation is not always suppressed by cultural conditions, which can also produce cells of different physiological age. Singh (personal communication) has failed to correlate fimbriation and invasiveness in similar experiments using three different non-fimbriate strains and their fimbriate transductants. The use of transductant pairs effectively eliminates the possibility of strain differences other than fimbriation being involved. In view of these criticisms it would seem that Campbell's conclusions are not valid, and so do not contradict the hypothesis that fimbriation is not an invasive aid. Duguid and Gillies (1957) concluded that, when present in pathogenic organisms, fimbriae subserve some other role. When pathogenic organisms such as Shigella flexneri are fimbriate, it is thought that the non-fimbriate form is responsible for the invasive stage, and that the fimbriate form satisfies a commensal, surviving

role. The commonest bacterial plant pathogens belong to the genera Erwinia (which is closely related to Klebsiella), Xanthomonas and Pseudomonas, and, of course, fimbriae have been demonstrated in saprophytes of closely related genera. Soft-rot of storage organs and vascular infection of the conducting vessels are the common manifestations of plant disease. The presence of fimbriae to ensure continued contact with readily available nutrients would be of great advantage in species survival. An objective search among the plant pathogens for the presence of fimbriae and their possible role in plant pathogenicity would seem worthwhile.

3. Pellicle-forming ability.

Duguid and Gillies (1957) showed that in aerobic static broth, fimbriate cells were selected apparently by their ability to concentrate at the air-broth interface by forming a thin pellicle. Duguid and Wilkinson (1961) show comparative growth rates for the fimbriate and non-fimbriate phases of Shigella flexneri under three different environmental conditions. Anaerobic and rotated broths follow essentially the same growth pattern, while in aerobic static broth the fimbriate strain undergoes a phase of secondary growth concomitant with the formation of a characteristic pellicle. Worthy of consideration in conjunction with these observations is the finding that the mean generation time of two pure phases of E.coli B in tryptone broth at 37° was 30.5 min. for the fim ơ+ type and 29.0 min for the fim ơ- type (Brinton, 1959). Although no specific investigation

has been made relevant to the generation times of other kinds of fimbriate cells, such a difference between the fimbriate and non-fimbriate phases of fimbriate cells is probably also true (see, for example, figure 2). This difference in growth rates though small, would quickly lead to a relatively great increase in the population of non-fimbriate cells, with the result that the additional burden imposed on a cell by the synthesis of fimbriae would eventually lead to the elimination of fimbriate cells. At a time when the situation is becoming critical, however, fimbriate cells are able to counteract this faster growth rate of the non-fimbriate cells by an outburst of secondary growth. Pellicle-forming ability is, therefore, of considerable importance in survival.

Presumably organisms growing in the pellicle have increased oxygen availability and the non-fimbriate cells growing in the anaerobic depths will be killed by the toxic waste products of the other. The fact that fimbriate and non-fimbriate forms grow equally well under conditions preventing pellicle formation supports this hypothesis. The finding that fimbriate cells are viable under conditions favourable to their formation, argues that they are not the pathological end-products of unfavourable environmental conditions, although such a conclusion was necessary to account for the role suggested by Houwink and van Iterson, who considered that fimbriae might be excretory products produced by resting cells. Pellicle-forming ability must

be excluded as contributory to the promotion and establishment of an enteropathogen in its natural habitat, where the conditions of aerobic static culture are far from simulated. However, the advantages gained from this ability are obviously important in the survival of saprophytic organisms, such as Cloaca cloacae, Escherichia coli and Proteus, in their natural habitats where oxygen is limited.

4. Protection against bacteriophage.

Brinton et al (1954) claimed that fimbriate E.coli strains were less susceptible to infection by T phages than the non-fimbriate form, and that a fimbrial suspension inactivated these phages. On the other hand, Duguid and Gillies (1957) found no proof of a phage-protective role in Shigella flexneri strains. The finding of the former group was accounted for by identifying the spheroidal surface material contaminating the fimbrial preparation as similar to that isolated in pure form and shown to be the receptor sites for T phage adsorption (Weidel and Kellenberger, 1955).

5. Association with fertility.

It is known that the fertility factors of E.coli determine the presence of surface antigens (Maccacaro and Colombo, 1956, Ørskov and Ørskov, 1960) and that F+ and Hfr cells possess a surface component probably responsible for the adsorption of male to female cells during the mating process (Maccacaro, 1955, Sneath and Lederberg, 1961). From the work of Maccacaro, 1955, and Maccacaro and Comolli,1956, it emerged that fimbriae were not the morphological

equivalents of fertility at the cell surface, determining adhesion during the mating process. This was confirmed by Brinton, 1959.

Fimbriae have no known association with the other common episomes, prophage or colicinogenic factors. Lysogeny and colicinogeny occur quite independently of fimbriation in salmonellas (Duguid, personal communication). Nevertheless, a relationship between the colicinogenic factors and fimbriae might have been suspected from the finding that fimbrial preparations possessed colicin E activity (Brinton, 1959). This was a coincidental association of two unrelated properties, however, because purification of the fimbriae did not increase antibiotic activity.

6. Association with nutritive functions.

Maccacaro and Angelotti (1955) calculated that the presence of fimbriae on a cell did not greatly increase the cell volume, but more than doubled its surface area. They suggested that this might be of nutritional importance, an idea originally expressed by Houwink and van Iterson (1950), who thought that fimbriae might be the organs of nutrient uptake. This would demand that they were highly selective in action, however, because of the high concentrations of waste products present in most environments. Their absence under certain environmental conditions where their presence would be equally vital and advantageous, such as shaken, aerated and anaerobic broths, places this theory in doubt.

Maccacaro and Angelotti (1955) had also found that loss of fimbriae in certain E.coli strains was associated with a loss of fermentive capacity. This was more fully investigated by Dettori and Maccacaro (1959), who studied the respiratory activities of recombinant fim +, fim (+) and fim- types in broth cultures, and revealed a notable difference between fimbriate and non-fimbriate cells. Fim + cells had a greater oxidative ability than fim - cells, and phenotypically non-fimbriate fim (+) cells behaved as fim - cells. A similar effect was found by the addition of Tween 80 (0.05%) to the broth, or if the broth was replaced by a minimal medium, each of which is claimed to suppress pellicle formation. However, it has been found that non-lethal concentrations of Tween 80 (as high as 0.2% final concentration in broth) do not completely supress the pellicle-forming ability of a fimbriate cell (Contento and Old, unpublished results). They also found that the glycolytic activity of fim - and fim (+) cells was greater than that of fim + cells, and that the addition of glucose to the broth caused the fim + type to behave as if non-fimbriate because this suppressed their pellicle-forming activities.

These conclusions were confirmed and expanded in an investigation of the utilisation of carbohydrates and Krebs-cycle intermediates, again using recombinant isolates. (Maccacaro and Dettori, 1959). They showed that anaerobic glycolysis proceeded more rapidly with fim - than with fim + cells, all the glucose being converted to lactic acid in one

hour. The oxidative activity of fim + cells was greater than fim -, and in both cases fim (+) and fim - cells behaved similarly. The validity of comparing the fermentations of fim + and fim (+) phases seems questionable, since differences in fermentation capacity could well be caused by physiological differences in the strains arising from different cultural conditions as well as differences in their fimbriation status. Nevertheless, these results have been repeatedly concluded as significant of the selective advantages bestowed on the two kinds of cells. The greater oxidative ability of fimbriate cells fits in well with their greater growth potential under certain aerobic conditions, and it has been suggested that non-fimbriate cells were better adapted for growth under anaerobic conditions by virtue of their greater glycolytic activity (Duguid and Wilkinson, 1961).

A completely different picture, however, has been presented by other workers. Downie (unpublished results), working with E.coli strains K12d4 and K12βI used by Maccacaro and Dettori (1959) was unable to confirm their findings. She showed that strain K12βI, supplied as non-fimbriate, gave weak mannose-sensitive haemagglutination, but did not prove the presence of fimbriae electron microscopically. This could obviously account for some discrepancy in the results. From her investigations with these and other strains, it emerged that differences in fermentative ability could be related often to different viabilities in

the compared strains, a control apparently not tested by Maccacaro and Dettori. Comparison of a non-fimbriate Salmonella typhimurium strain and its fimbriate transductant showed them to have similar respiratory activities. Confirming Downie's findings, Wohlheiter, Brinton and Baron (1962) were also unable to differentiate fimbriate and non-fimbriate variants of E.coli BL(E) and E.coli Bam by their oxidative or glycolytic abilities. They also showed that the parents and hybrids from an "in vivo" cross between E.coli and S.typhimurium had different rates of utilisation of all the substrates tested except glucose, and that this difference was due to the spontaneous origin of mutants (at a frequency of 10^{-5} to 10^{-4}) capable of utilising the tested substrate. They concluded that any differences in respiratory activity between fimbriate and non-fimbriate cells can be explained by assuming that the two properties, fimbriation and respiratory activity, segregate independently in genetic crosses by conjugation. Maccacaro and Dettori (1960) found that fimbriated cells were agglutinated by a wide variety of dipolar molecules (dicarboxylic acids, diamines and monoaminocarboxylic acids); some of the substrates used in their experiments on respiration were, of course, dipolar molecules. The suggestion that the respiratory differences between fimbriate and non-fimbriate cells found by Maccacaro and Dettori might be explained in terms of their differential agglutinability by dipolar molecules is not valid in light of the findings by Downie

and Wohlheiter *et al*. One drawback in the acceptance of the conclusions of Maccacaro and Dettori is that they used recombinant pairs which might have differed in other unrecognised characters since recombination by conjugation can donate a considerable portion of the donor genome. It is not clear if Maccacaro and Dettori strains were interrupted immediately after the donation of the *fim* gene. If this were so, the hypothetical strain differences in recombinants might not be too great, since *fim* enters very early. Nevertheless, the available evidence would seem to favour the findings of Downie and Wohlheiter *et al*, who based their conclusions on fimbriate and non-fimbriate strains derived by transduction and mutation, each process involving the alteration of restricted portions of the chromosome, and so ensuring homogeneity of strain characters in all except fimbriation. The correlation between fimbriation and metabolic activity, if such exists, is not an obvious one on consideration of the available data.

Haemagglutination and red cell specificity.

Duguid *et al* (1955) observed haemagglutinating activity in a wide range of bacterial species; *E.coli*, *E.freundii*, *Cloaca cloacae*, *Alkaligenes faecalis*, *Salmonella typhi*, *S.paratyphi*,B., *S.enteritidis*, *Shigella flexneri*, *Sh.sonnei*, *Pseudomonas pyocyanea*, Proteus, *Bacillus cereus*, *Corynebacterium diphtheriae*, *C.hofmanni*, *C.pyogenes*, *Pasteurella septica*, *P.pseudotuberculosis*, *Vibrio cholerae* and the pneumococcus, but suggested that a common

haemagglutinating agent would be unlikely in all these species. Fimbriae were demonstrated only among certain of the gram-negative organisms. Neter (1956) reviewed haemagglutination phenomena and in bacteria classified two broad groups (a) that due to haemagglutinins which were diffusible and (b) that due to cell-bound haemagglutinins, causing direct bacterial haemagglutination. It is with the second group that we are concerned. In the E.coli strains they examined, Duguid et al (1955) demonstrated two kinds of cell-bound haemagglutination,(i) haemagglutination in the majority of strains was caused by fimbrial haemagglutinins (ii) three non-fimbriate strains showed haemagglutination due to a cell-bound haemagglutinin. In E.coli, Sh.flexneri, and C.cloacae fimbrial haemagglutination has the following properties (i) it is strongest with guinea-pig, horse, mouse and fowl cells and inactive for ox cells. Human and sheep cells are normally only weakly agglutinated (Duguid et al, 1955, Constable, 1956, Duguid and Gillies, 1957)., (ii) it is inhibited by D-mannose or α-methyl mannoside (iii) but not by an increase of temperature up to 55° (Duguid and Gillies, 1957). Fimbrial haemagglutination can, therefore, be readily differentiated from the somatic haemagglutinin - this is mannose resistant, elutes when warmed and shows strongest activity for ox, human and sheep cells - it has been designated the MRE haemagglutinin (Duguid and Collee, 1959). The adhesins responsible for haemagglutination are designated MS if their activity is inhibited by mannose

and MR if resistant. MS adhesins, associated with fimbriae, are the commonest, but not the only cause of haemagglutination in enterobacteria.

Duguid (1959) discovered some fimbriate strains of Klebsiella which, in addition to the typical MS haem-agglutination, were capable of agglutinating ox red cells treated with tannic acid, red cells heated at 70°, fungal mycelia and plant root hairs. These MR adhesins whose agglutinating activities, as the name suggests, were not inhibited by the presence of mannose, were associated with the presence of fimbriae. The majority of strains possessed either MS or MR adhesins, and a minority of strains showed both types. Shedden (1962), investigating the fimbrial adhesins of 79 Proteus hauseri strains, found that they were unaffected by mannose. This fimbrial haemagglutinin, though mannose resistant, was different from the MR Klebsiella haemagglutinin since it strongly agglutinated untreated ox and sheep cells. It has been designated the MRP haemagglutinin. In the closely related Providence group, Wright (unpublished results) has found a mixture of MS and MR haemagglutinins, which, unlike the Proteus fimbriae, do not show a phase variation with cultural conditions. These detailed descriptions of red cell specificity, while being useful diagnostic tools for the classification of the different types of fimbriae, are of no significance in relation to specific adhesive properties to selected substrates of a fimbriate organism in its natural

environment. Knowledge of the structure of fimbriae or the active sites present on the substrates which they agglutinate is hardly likely to emerge from such studies, in view of the fact that the active site for fimbrial action is present on such a wide range of cell varieties.

Fine structure of MS and MR fimbriae.

A knowledge of the fine structure of the different kinds of fimbriae emerged only from direct observation with electron microscopical techniques. The first indication of morphological diversity between the two types came from the investigations of Duguid (1959). He suggested that shadowed preparations of MR fimbriae in some Klebsiella strains appeared thinner, 7 mμ, than the normal MS fimbriae which he calculated as being 10 mμ in diameter. The realisation that shadowing increases the detail but limits resolution caused him to reserve judgment, and suggest that this apparent difference might be an artefact inherent in the preparation of his specimens. And yet, other differences became apparent between the two types. For example, Old (unpublished results) showed that the haemagglutinating activity of thin fimbriae could be removed by trypsination, which did not affect the MS fimbriae. The discrepancy between the two was clarified by Thornley and Horne (1962) who stained thick and thin fimbriated strains of Klebsiella by the "negative staining" technique with phosphotungstic acid (PTA). Their results conclusively showed that the 200 - 400 thick fimbriae per cell had a diameter of 65 - 70mμ,

while the 400 - 700 thin fimbriae per cell had a thickness of only 4.8 mμ. Their work further revealed that the two types of fimbriae were resistant to prolonged autolysis. A preparation of Proteus, after 20 days at 20°, showed the presence of flagella and fimbriae. After three months at 37°, the cell walls had disintegrated into rounded fragments, the flagella had disappeared but large numbers of fimbriae remained. One of their electron micrographs, (Plate 2, fig. 5.), suggests a regular periodicity of 4.5 - 5.0 mμ alongthe filament axis of MR fimbriae. The small component spherical units were thought to be arranged as threads, less tightly packed than the MS fimbriae. This excellent description of the fine structure of MR fimbriae was followed by an even more detailed interpretation of the more common MS fimbriae by Brinton and his coworkers.

Brinton and Huang (1962) claimed that fimbriae were composed of regularly arranged subunits. Various aggregates were considered evidence for a regular substructure within a single filament. Agents such as 6 M urea, glacial acetic acid, 1.5 M guanidine HCl, and 1.5 M sodium salicylate, which break the weak bonds maintaining protein structure, were shown to disintegrate fimbriae completely, as evidenced by electron microscopy. Brinton (personal communication, 1963) also examined purified suspensions of fimbriae from E.coli Bam P + by the techniques used by Thornley and Horne. He found that fimbriae aggregated to form single layers or bundles of parallel fimbriae, with a centre-to-centre distance

of 6.2 - 6.4 mμ, i.e. 11% less than the diameter of a single filament (6.9 - 7.2 mμ). Obliquely orientated alternating layers of fimbriae had an angle of 41 - 42°. From his electron micrographs and calculations, Brinton postulated a model for fimbriae consisting of protein subunits polymerised in a right-handed double helix, diameter 70°A, an axial hole of 2.0 - 2.5 mμ and a pitch of 9.5mμ. The axial repeating distance of the subunits of the MS fimbriae was calculated as 4.99 mμ. Kerridge et al (1962), in the course of a study on the fine structure of the flagella of Salmonella typhimurium, encountered other appendages, obviously fimbriae, which they describe as filaments of diameter 5.0 mμ, showing no subunits. Their impression of long hollow cylinders is confirmatory of Brinton's finding of an axial hole. One surprising point, however, is their measurement of the diameter as 5.0 mμ which is more in line with Thornely and Horne's estimate for MR fimbriae. Salmonella typhimurium, of course, has MS fimbriae whose diameter is of the order of 7 mμ.

Chemistry.

Weibull and Hedvall (1953) attempted to analyse fimbriae from Proteus strain, X19H. By shaking with glass beads for three hours, they obtained a suspension which could be fractionated by differential centrifugation. One of these fractions contained both flagella and fimbriae. Adjusting the pH removed the flagella and the fimbriae were thus isolated. When extracted with 0.55 N NaOH at 20° for

three hours, the filaments showed only a weak absorption in the ultraviolet range. Neither were they destroyed by N NaOH or N HCl at room temperature, nor by the enzymes trypsin, pepsin, ribonuclease and deoxyribonuclease, as evidenced by their anatomical appearance in the electron microscope. The authors concluded that chemically the fimbriae were composed of rather inert material and that structurally they formed part of the cell walls.

Brinton (1959) analysed the fimbriae of E.coli BL(E) obtained after two minutes "shaving" in a Waring blendor. As seen by electrophoresis, the resulting suspension separated into two components. The leading boundary was of pure fimbriae, the trailing one of spheroidal material. Electrophoretically purified fimbriae had a nitrogen content of 4.6%, and gave positive xanthoproteic, biuret and weak ninhydrin reactions. They had a peak in the ultraviolet range at 279 mμ and were precipitable by ammonium sulphate. Their morphological resistance to extremes of pH and temperature confirmed the work of Weibull and Hedvall (1953) for Proteus fimbriae and Duguid and Gillies (1957) for Shigella fimbriae. It was concluded that they were not pure protein. Two years later, a short communication on the chemical nature of fimbriae was made by Brinton and Stone (1961). They detached, concentrated, and purified fimbriae from E.coli Bam by isoelectric precipitation at pH 4.0. Resuspension at pH 7.0 and standing in the cold caused the formation of "paracrystals" which could be

centrifuged at low speed from contaminating material. This method had apparently not resulted in disaggregation of the fimbriae, since electron microscopical observations refer to pure fimbriae and make no mention of loss of morphological form. These paracrystals, therefore, represent bundles of fimbriae and not fimbrial sub-units. Tests for RNA, DNA, phosphorus, polysaccharide and reducing sugars in their pure preparations were each less than 0.6%. The purified fimbriae, judged to contain at least 98% protein, showed typical protein spectra in the ultraviolet and infrared ranges. However, the Lowry and ninhydrin assays used are based on the presence of certain bondings and amino acids, and their reliability as quantitative assays varies with the protein concerned. Their protein estimates would have been more satisfactorily judged by a total nitrogen estimate as used in the previous paper.

They also showed that the optical density of purified fimbriae was decreased by trypsin and pepsin. The observation of a decrease in optical density of fimbrial preparations after treatment with pepsin and trypsin is not significant proof of their lability to these enzymes in the absence of electrn microscopical observations. In fact, such a finding would be in complete contradiction to the electron microscopical observations made by Weibull and Hedvall (1953). It seems that this decrease represents a further removal of residual contaminating material present in their preparations. Brinton's (1959) estimation of a

total nitrogen content of 4.6% suggested a protein content of 30%, which, in view of his later findings of approximately 100% protein, indicates a considerable contamination of his electrophoretically "pure" fimbriae. The most likely contaminant would probably be cell wall material which exerts a considerable masking effect on an ultraviolet spectrum. It is, therefore, surprising that these impure preparations showed such strong absorption at 280mμ. It would also seem to indicate that even electrophoresis and electron microscopy in conjunction are not adequate criteria of purity. Brinton (personal communication, 1963) has extended these observations and characterised fimbriae as pure protein. He has also shown a typical range of amino acids with a preponderance of acidic amino acids, which must be meutralised by high concentrations of divalent cations to allow haemagglutination to occur.

Antigenicity.

The first detailed antigenic studies on fimbriae were made by Gillies and Duguid (1958). They found that fimbrial antigens could be distinguished from all the other surface antigens since antisera prepared against non-fimbriate strains showed no reaction with fimbriate ones, and distinguished fimbrial from flagellar antigens, since the latter was destroyed by 0.005 N HCl or 50% ethyl alcohol. Fimbrial antigens have been found to be somewhat resistant to most degradation processes, e.g. their morphology and antigenicity remain intact even after heating for one hour

at 100°, and not until they have been heated at 100° for 150 min. are these properties lost. Fimbriae can be readily detached from bacteria, 90° for 30 min. or 100° for 2 min., but this was a confusing factor since it did not signify loss of activity of the fimbriae.

Gillies and Duguid (1958) obtained pure fimbrial antiserum against Shigella flexneri by absorbing from the crude fimbriate serum, the common antigens found in the non-fimbriate, phenotypically-repressed strain. This specific anti-serum agglutinated fimbriate Shigella flexneri, but not the non-fimbriate strain, and inhibited the haemagglutinating activity of fimbriate shigellas. Using such absorbed antifimbrial sera, they were able to show that all strains of Shigella flexneri, irrespective of their O-serotype, had fimbriae of the same antigenic composition. Brinton (1959), using an antifimbrial serum prepared against electrophoretically "purified" fimbriae, confirmed some of their results. It would seem, therefore, that fimbrial antigens are not subject to the degree of variation characteristically found in flagella. Gillies and Duguid (1958) showed some cross-reaction between the fimbrial antigens of Sh.flexneri and E.coli. All cross-reactions were at low titre except that by E.coli 253, which absorbed all the antibodies from an antifimbrial Sh. flexneri serum. One or more minor flexneri-coli fimbrial antigen determinants were shared with most E.coli strains, and another with a few strains of group 51. There appeared to be no cross-reaction between Shigella

flexneri and fimbriate strains of Salmonella (S.paratyphi B, S. enteritidis, S.typhimurium and S.thompson, Cloaca cloacae and Proteus. The antigens associated with fimbriae seem to be genus-specific and might be of value in the classification of bacteria.

The genus specificity of fimbrial antigens was confirmed in Salmonella (Campbell, 1961). In this investigation, antifimbrial sera were prepared against the fimbriae of 25 strains of 22 serotypes. By cross-agglutination tests with absorbed sera, he distinguished five fimbrial antigens; some strains were monoantigenic, e.g. S.moscow, f.a.I, while others contained up to three antigens, e.g. S.typhimurium, f.a. I,2,3. There were many degrees of sharing, e.g. S.typhimurium (I,2,3), S.enteritidis (I,2,5), S.pullorum (I,5) and S.abortus-equi (I). This scheme, however, has one drawback. Preparations of antifimbrial sera by absorbing out the common antigens present in a phenotypically non-fimbriate strain is not valid unless one can be sure that cultural conditions definitely eliminate all traces of fimbriae (and this would seem a difficult demand). Traces of fimbriae would remove significant amounts of fimbrial antigens since this method required repeated absorptions with phenotypically non-fimbriate cells at a high cell density. Again, different cultural conditions might repress somatic antigens other than the fimbrial ones. Brinton (personal communication) subjected permanently fimbriate and non-fimbriate strains of E.coli Bam to the

same purification method for the isolation of fimbriae (isoelectric precipitation) and found that large amounts of extracellular material other than fimbriae were produced by the fimbriate strains and not by the non-fimbriate. If this were true for other species, then the antifimbrial serum obtained by the absorption technique will contain antibodies other than those due to fimbriae. This might invalidate Campbell's results, if the non-fimbrial material produced by different species was not chemically the same. Certainly, his scheme would have been less open to criticism if the fimbrial antiserum had been prepared against purified fimbriae. Since no such purification method was then available, this is an unfair criticism against otherwise good work. This inadequacy has since been corrected and it is to be hoped that similar work will be attempted using purified fimbrial preparations.

Other antigens in enterobacteria have been described in the literature which might have been fimbrial, e.g. the antigen α (Stamp and Stone, 1944), the β antigen (Mushin, 1949), the T antigen (Stuart, Feinberg and Feinberg, 1948) and the X antigen (Topley and Ayrton, 1924). The relationship of these four antigens is discussed by Gillies and Duguid (1958); suffice it to say that of these, the X antigen is most like the fimbrial antigen, since it is theremostable (100° for 2 hr.) gives rapid agglutination in tubes within 2 hr. at 37° and its presence fluctuates with the same environmental conditions controlling phase variation.

Genetics.

The genetic control of fimbriation will, no doubt, prove as complex and interesting as that of flagella. Indications of chromosomal control of fimbriation have been found, and the non-essential nature of fimbriae as cell components and the high rate of phase variation are suggestive of some additional control by a determinant, possibly of episomic nature. For example, Duguid and Wilkinson (1961) suggested that fimbrial phase variation involved spontaneous changes in a heritable determinant, and the environmental selection of mutants. This was hypothetically based and no proof of this determinant exists. If, on the other hand, it were imagined that the phase variation was a direct effect imposed by the environment, then such expression should manifest itself rapidly.

As well as the fimbriate type which becomes non-fimbriate under certain cultural conditions, permanently non-fimbriate types are known. Maccacaro, Colombo and Di Nardo (1959) showed that 4 out of 61 strains of E.coli were irreversibly non-fimbriate and could become so only by recombination. The locus controlling fim was found to be close to the thr-leu region, because in fim + F + x fim- F - crosses, 70 - 80% of the recombinants selected for thr or leu were fimbriate. They suggested that a system of plasmagens, transferrable during mating, in conjunction with the chromosomal gene, controlled the production of fimbriation, and the expression of the character, since

the latter was correlated with the degree of phenotypic expression in the fimbriate donor. Additional findings claimed to justify this theory were that certain fimbriate recombinants become irreversibly non-fimbriate after a few subcultures, and that irreversible loss of fimbriation on subculturing at 42°. They were unable to map fim on the chromosome since there was ~~no~~ direct correlation between the length of the fim + donor genome inherited and the frequency of fimbriation among the recipients. Certain anomalies in the distribution of fimbriation in fim+ F+ x fim- F- crosses might be explained by Downie's finding that E.coli K12βI showed haemagglutinating activity. However, the same anomalies were later found in other crosses (see Maccacaro and Hayes, 1961a).

Brinton, Gemski, Falkow and Baron (1961) used an interrupted mating technique with suitably marked E.coli strains to show that fim was situated to the left of thr, ara and leu on the chromosome map. Results, using phages PIkc and PIbt showed that some of the transductants selected on threonine, arabinose or leucine media were fimbriate. The unusually high linkage with known chromosomal markers suggested that there might be a second fimbriation determinant, capable of attachment to the sites of the other three markers. This might be an episome. (Brinton, et al, 1961, Brinton and Gemski, 1961). However, the cotransduction rates with these other markers are unusually high being 30%, 28% and 13% for threonine, leucine and

arabinose respectively. Although the biochemical markers were transduced to give stable progeny, all fimbriate cotransductants segregated non-fimbriate cells immediately. The unusually high frequency of fimbriation found among the cotransductants could be more satisfactorily interpreted by assuming that back mutation to the fimbriate state had occurred. Maccacaro and Hayes (196la,b) presented a detailed analysis of their investigations into the genetic control of the different types of fimbriation found in E.coli. Using well-characterised donor and recipient strains, they were unable to map fim by linkage data using uninterrupted mating, but proceeded to do so by an interrupted mating procedure. They showed that in experiments with donor Hfr H strain that fim enters the recipient cell 2 - 3 minutes ahead of thr. Fim was placed close to thr, about one quarter of its distance from thi. Their chromosomal map, showing distances between markers in time units, was as follows:-

-met --- thi --- fim --- thr-leu --- az ---
2 6-7 2-3 I 0.5

To investigate any possible differences, chromosomal, cytoplasmic or episomic, between fim + and fim σ+ donors, these were crossed with fim - F - recipients. They found that the phenotypic state of the fim + cells did not affect the efficiency of recombination, both giving fim + and fim - cells at the same frequency. Fim σ+ donors gave recombinants which were either fim σ+ or fim - (although

transduction experiments with fim σ+ donors had occasionally given fim + transductants, a finding suggestive of a distinct locus σ closely linked to fim.) Fim - and fim σ= strains of E.coli were used as recipients with fim + dnors, revealing that fim cells in recombinant crosses never gave fim σ+ types, although fim σ= recipients gave an average of 8% fim σ+ recombinants. This suggested that, genetically, fim σ= cells were different from fim -, and that the fim σ= type had some additional determinantnabsent from fim - (presumably, a part of the hypothetical σ locus.). This genetic difference between the two types of irreversibly non-fimbriate cells had not been apparent when these were used as donors in crosses with a fim + F- recipient, since neither cross yielded fim σ+ recombinants. They suggest and discuss three models for the production and type of fimbriation; the best, involving an episome and a structural gene is, however, inadequate. They point out that they have examined only one type of each of the three kinds of cells - fim σ+, fim σ= and fim -.

It would seem a difficult task to differentiate between the two types of non-fimbriate strains, namely fim σ= and fim -, and the only criterion available is one based on different respiratory activity (see table I of Maccacaro and Hayes, 1961a) a property about which disagreement exists. Wohlheiter et al (1962) expressed their inability to obtain a permanently non-fimbriate form of E.coli K12-58:161 and, indeed, the Q_{O2} and Q_{CO2} values

quoted for the fim 6+ and fim 6- types are identical (Maccacaro and Dettori, 1959, quoted in table I, Maccacaro and Hayes, 196la), a fact which would agree with the claims of the former group. It is unfortunate that so much of the painstaking analysis reported by Maccacaro and Hayes is now open to doubt in view of the obvious disagreement of these two research groups about the types of fimbriation to be found in this one strain. It is to be hoped that these difficulties can be overcome by similar analyses with other strains.

Classification and Epidemiology of Salmonella typhimurium.

Salmonella typhimurium is classified in the Kauffmann-White scheme as a Group B organism, (I), IV, (V), XII:i:I,2,3. It has fimbrial antigens 1, 2 and 3 (Campbell, 1961). Salmonella species generally do not ferment sucrose, lactose, adonitol or salicin, but ferment glucose, mannitol, dulcitol, sorbitol, maltose and dextrin. Arabinose, xylose, rhamnose, trehalose and inositol are the sugars most useful in species differentiation. The organic acids, d-tartrate, l-tartrate, citrate and mucate, are useful adjuvants whilst most species ferment Stern's glycerol fuchsin broth (1916) and most grow in Bitter's minimal medium (1926).

In Salmonella typhimurium, strains identical serologically can often be differentiated by their fermentation reactions. On the basis of fermentation and cultural reactions, it has been possible to subdivide Salmonella typhimurium into types. For example, Kristensen, Bojlen and Faarup (1937) established eighteen such fermentation-types in Salmonella typhimurium. (These fermentation-types were later expanded by Harhoff, 1948). These "biochemically" established types are usually stable enough to be of epidemiological value. For example, strains isolated from ducks and chickens in Europe are usually rhamnose, inositol and citrate-negative, whilst those from American ducks are usually rhamnose-fermenting (Edwards and Bruner, 1940). Typing on such a basis, although giving information of

epidemiological significance, is usually too time-consuming in investigations on outbreaks of typhimurium infection. With respect to their fermentation of rhamnose, Kristensen (1944) showed that there were two distinct types. The first group fermented rhamnose strongly and quickly, while the second group attacked it more weakly and late. This was called a "mutative" reaction. In the course of eleven years, he isolated a number of strains from different countries. Twenty-two of these strains did not ferment rhamnose within twenty-four hours, five gave late fermentation in three to five days, and seventeen did not ferment it in up to thirty days. The majority of the late rhamnose fermenting strains he found to be also late tartrate-fermenters:-

Rha - Inl - Tartrate + Stern + = 2
Rha - Inl - Tartrate - Stern + = 14
Rha - Inl - Tartrate - Stern - = 5

Kauffmann (1954) examined 85 strains of Salmonella typhimurium and found that 14 of the 85 strains (i.e. 16.5%) were inositol non-fermenting and 10 out of 85 strains (i.e. 12%) were late rhamnose fermenters or did not ferment this sugar at all. Most strains fermented xylose and trehalose promptly, and the significance of xylose late-fermentation in epidemiology is noted (Harhoff, quoted in Kauffmann, 1954).

Duguid (unpublished results) has shown that among Salmonella typhimurium strains, the characters of rhamnose and inositol fermentation (i.e. the ability to ferment these

sugars promptlyin sugar peptone water and also to utilise it as sole carbon and energy source) and fimbriation (i.e. the ability to produce the non-flagellar, surface appendages, called fimbriae) were all closely related. His results show that among more than 600 strains, approximately 80% of the strains examined were fim +, rha + and, commonly, inl + and 20% were fim -, rha -, almost always, inl -, although one strain, Sa 6925, is inositol-fermenting. These strains had been isolated from various sources of human and animal infection from many countries over a period of ten years. A vast number of different phage-types and fermentation-varieties was noted within the strains, each normally being epidemiologically unique. Thus, the earliest surveys and the most recent were significant in their correlation of the characters. This absolute correlation, especially of the characters of fimbriation and rhamnose fermentation, being absent or present together, is suggestive of the two being genetically controlled at a single locus or at very closely linked loci.

Unpublished data (Duguid, personal communication) have shown that in Salmonella typhimurium, rhamnose fermentation was of two kinds. Strains belonging to the fim + inl + rha + (FIRP) group were of the strongly-fermenting kind. They fermented rhamnose in 1% rhamnose peptone water within six hours, produced red-black colonies on rhamnose-eosin-methylene blue (REMB) agar and utilised rhamnose as the sole source of carbon and energy on minimal medium supplemented

with rhamnose in 48 hr. All fim - inl - rha -(FIRN)strains of S.typhimurium have been shown, when grown in rhamnose peptone water for times from three to twenty-one days, to produce mutants capable of fermenting rhamnose. When isolated in pure culture, these late-fermenting mutants ferment rhamnose within 24 hr, but not in 6 hr. as the FIRP strains do. Although fermenting rhamnose in peptone water, these late mutants do not grow on minimal rhamnose medium and are unable to ferment rhamnose in REMB medium. A similar inability to utilise a sugar as a carbon and energy source in minimal medium and yet acidify in sugar peptone water was shown by 21 strains of Bacterium anitratum with the sugar lactose (Villecourt and Blachere, 1955). It is not a common phenomenon.

Inositol late fermenting strains have very rarely been spontaneously isolated from FIRN strains of S.typhimurium when grown in inositol peptone water for up to 28 days. This may be because inositol is not a good carbon and energy source.

Salmonella typhimurium is a natural rodent pathogen, but has been isolated from guinea pigs, sheep, cattle, pigs, cats, foxes, pigeons, parrots, canaries, penguins, turkeys, chicks and ducks. (Miles and Wilson, 1955). A large number of isolates are the known causative agents in dried egg, resulting in food-poisoning or typhoid-like diseases in man. Apart from these animal reservoirs, food, water, eggs and sewage have all been quoted as foci of Salmonella

infection. Edwards and Bruner (1943) found that of 2,520 cultures of Salmonella examined, 1258 were from fowls, 532 from man, 475 from swine, 90 from rodents, 88 from carnivores, 53 from horses and 20 from ruminants - (i.e. 2,516). Of those from man, Salmonella typhimurium was the second most common after Salmonella paratyphi B. Salmonella typhimurium (aertrycke) is the organism most commonly isolated from food poisoning outbreaks, in Great Britain and U.S.A.

In outbreaks of S.typhimurium infection, the source may be traced to infected animals of any of the above numerous species. In order to trace these outbreaks quickly and accurately, attempts have been made to classify S.typhimurium into sub-groups. We have already discussed the fermentation groups of Kristensen et al (1937) and Harhoff (1948). A limited differentiation is also possible on the basis of certain serological characters, since antigenic variants are known. S.binns is a type lacking the phase I,i, flagellar antigen. S.copenhagen (storrs) lacks O-somatic antigen V. Nearly all isolates from pigeons lack O-somatic antigen V, which allows the establishment of a "pigeon" type of S.typhimurium. The isolation of such factor V-negative variants usually suggests that such infection can be traced directly or indirectly to a focus of infected pigeons. Epidemiological typing on either of these bases is, however, complicated and time-consuming. Another valuable aid to the epidemiologist in tracing the

paths of an infection is phage-typing. This is simple, efficient and speedy, being complete within 24 hr. Several successful typing methods are available for S.typhimurium. Lilleengen (1948), using a typing method based on the use of a selected combination of twelve anti-O (non-specific) phages, was able to type about 95% of 703 strains of S.typhimurium into 24 phage-types. These types were stable "in vivo" and "in vitro". Six of the 24 types were unique to isolates from man, and seven to isolates from animals and egg-products. Some types were recognised as species-specific, e.g. type 3 from ducks and geese, type 9 was also a "bird" type, type II from rats, and types 6A and 6B from pigeons. Boyd (1950), by identifying the natural phages carried by a strain, also classified S.typhimurium isolates successfully. Another successful scheme makes use of adapted Vi phages of S.typhimurium (Felix, 1956, expanded by Callow, 1959).

CHAPTER III

Genetic variation in bacteria.

In bacteria, all the deoxyribonucleic acid of the cell is contained in a structure called the nuclear body, which corresponds to the nucleus of higher organisms. The many investigations of the genetic determinants in bacteria indicate that these can be mapped linearly along the chromosome in the form of a single, closed loop. DNA, according to the Watson-Crick model, is a linear sequence of nucleotide pairs in the form of a double helix, held together by weak bonds so that the guanine and thymine on one chain complement, respectively, the cytosine and adenine of the other. The genetic specificity, which is an inherent part of DNA, resides in the linear sequence of these bases along the chain, the exact sequence of which determines the amino acid sequence in proteins. "Messenger" RNA is accepted as being the agent responsible for transferring the information from the DNA to the ribosomes, the sites of protein synthesis. This reading of the bases along the chromosome, which controls the protein specificity, has recently been investigated more fully and discussed in terms of a "triplet code" (Crick, Barnett, Brenner and Watts-Tobin, 1961). In this, it is claimed that the reading of bases in triplets from a definite starting point determines the specificity of the proteins synthesised, each amino acid being coded by at least one triplet of bases. Any alternationin this sequence, by the

addition or deletion or substitution of a single base, either spontaneously or by the action of mutagens, may result in a mutation. This will be manifested either by the production of an atypical protein with different physical and chemical properties to the wild-type protein, or by the complete absence of any protein.

New genotypes, however, can occur by means other than mutation. In bacteria, recombination has been defined as the alteration of genotype resulting from the interaction of a micro-organism with any other organism, or with genetic material derived from that organism. Genetically-active DNA can be transferred from one bacterium to another by various methods - (i) by a mating process, conjugation, which is the only thing approximating to the sexual process in higher organisms, (ii) by a transformation process in which the bacterial traits of one organism are transferred to another by naked DNA, (iii) a transduction mechanism in which the DNA is again exchanged between organisms, this time being enclosed in a protective vector, bacteriophage, (iv) a process of infection in which traits are transferred by a class of genetic determinants, classified as episomes. A short review of the different mechanisms of transfer will now be given, which is not intended to summarise the many aspects of these divergent subjects. Neither conjugation nor transformation will be dealt with other than summarily, since these mechanisms were not relevant to this investigation. Rather, those which

are relevant to this research, by their use or by the necessity to consider them in the final analysis, namely, phage-mediated transduction and episomic infection, will be discussed in more detail.

1. Conjugation, is a phenomenon in which hereditary traits are exchanged between bacteria during a mating process, in which the chromosome of a male cell is injected, wholly or partially, into a female recipient cell. The process is dependent on the presence of fertility factors in a cell - these will be discussed further in the section dealing with episomes. One important feature of sexual recombination has been its demonstration "in vivo" between the two inter-fertile strains, E.coli and S.typhimurium (Schneider, Formal and Baron, 1961). After mice hadbeen fed these two strains separately on successive days, small numbers of hybrid forms were recovered from the faeces within twenty-four hours. Fertility could not be demonstrated "in vitro" between these two strains suggesting that the range of genetic transfer may be even greater than "in vitro" investigations are capable of revealing. (For reviews on conjugation, see Hayes, 1960 and 1962).

2. Transformation. Transfer of genetic characters from one cell to another by cell-free extracts was first demonstrated in Griffith's work in the transformation of pneumococci. The transforming principle was later isolated in highly-purified form and characterised as DNA. This was the first chemical proof that DNA acted as

genetic material (Avery, McLeod and McCarty, 1944, Hotchkiss, 1949). Transformation has been described in Salmonella strains, but has not been successfully demonstrated with other species of enterobacteria (Hartman and Goodgal, 1959). Its importance as a general and frequent means of genetic change is not readily assessable, but it might be imagined that this is probable during autolysis. It is well-documented that the efficiency of transfer is lower with transformation than other methods of DNA transfer. The many difficulties inherent in the preparation of pure DNA and the uncertainty of success make this a difficult method for general application. (For reviews, see Hartman and Goodgal, 1959, Ephrussi-Taylor, 1960, Jackson, 1962).

3. Transduction. In 1951, Lederberg, Lederberg, Zinder and Lively transferred genetic characters from strain LT-2 of Salmonella typhimurium to strain LT-22 by a filtrable agent produced as a result of phage stimulation. Zinder and Lederberg (1952) identified the filtrable agent as having all the properties of the phage itself, and called the process "transduction". Phage-mediated exchange in salmonellas occurs when phage grown on one strain, the donor, incorporates some donor DNA during its maturation stage. Grown in the presence of a second strain, the recipient, this results in a permanent change in the heritable state of the recipient. By a process of substitution and replacement of part of the recipient genome by that of the

donor, there is usually in transduction only one character incorporated per recipient cell. Transduction has been reported in Salmonella, Escherichia, Pseudomonas, Vibrio, Staphylococci, Proteus and Bacillus (Hartman and Goodgal, 1959).

The term general transduction is applied to those phage-host systems in which the phages involved are not attached at specific loci to the chromosome, which means that any marker has an equally good transductional potential, provided that a suitable selective medium is available. There are two kinds of general transduction (a) complete transduction in which the transducing particle or exogenote is incorporated into the recipient genome by a process of "crossing-over" (b) abortive transduction in which such incorporation fails so that the exogenote is not reproduced, resulting in only one daughter cell receiving the particle at each cell division. This was discovered by Stocker et al (1953) as "trail" phenomena in semi-solid gelatin agar, and by Ozeki (1956) who in reciprocal transductions with purine-requiring mutants, noticed that as well as normal, large colony transductants, "minute" abortive colonies were also present, carrying the exogenote in unilinear fashion. In both cases, the offspring exerts a weak prototroph state until diluted out during growth. The frequency of minute colonies is higher than large colonies, so that a high percentage of donor alleles is lost through not being incorporated.

In general transduction, the whole genome is potentially transferable, at a low rate of one transduction per 10^4-10^6 infected cells. The chance of simultaneous transduction is thus rare, the first reports being those of Stocker et al, (1953) for the segment controlling antigenicity and motility in Salmonella flagella. Lennox (1955) has shown in E.coli that only those markers known from conjugation data to be near-sited on the chromosome could be transferred simultaneously. The DNA of phage has been calculated as approximately one-hundredth the amount of the bacterial chromosome, which means that during lysis the bacterial chromosome must be fractured in many places to allow phage-carriage. This explains why only closely-linked markers are carried by the one phage, e.g. Demerec et al (1958) showed that all the tryptophane loci and the closely linked cys B locus were carried by a single transducing particle of phage P22. It has also been suggested that the fragmentation of the donor chromosome by phage during lysis occurs, not randomly, but at specific loci (Ozeki, 1959). Yura (1956) showed that doubly-defective mutants were always transduced by particles containing both the homologous sites absent in the recipient, which suggested specificity of the length of the chromosome carried. Certain deletion mutants are known which, although suitable as donors, do not function as recipients in transduction. The deletion mutant, extending over two sites, can only be transduced as a result of double

transduction.

The relationship between the donor and phage genes in the transducing particles is confused. Sometimes, as in the E.coli-PI system, phage functions are missing altogether from the transduced cell, which is both non-lysogenic and sensitive (Lennox, 1955, Adams and Luria, 1958). In other cases, the transduced bacteria are always immune and lysogenic, so that they have both an integrated bacterial gene and phage genome, It is still not known whether the same phage particle can transfer both the fragment of the host gene and phage genome. Generally, however, it is considered that transducing phages are defective and that transduction and lysogenisation are mutually exclusive characteristics of a phage particle.

A great deal of information is available from the study of another kind of phage-mediated transfer. The transducing phage contains genetic elements in which part of the phage genome has been replaced by part of the bacterial genome, so that only a restricted portion of the bacterial chromosome is transferable. There is detailed information on two such systems (i) in E.coli K12, the phage λ transduces a number of genes controlling galactose fermentation, since the site of attachment to the bacterial chromosome is at the gal region for λ prophage, (ii) that of the lac loci with phage PI in E.coli (Morse, Lederberg and Lederberg, 1956, Lederberg, 1960, Luria,

Adams and Ting, 1960). Unlike general transduction, restricted transduction occurs only with phage lysates prepared by induction of the prophage. Transduction by phage λ of the gal genes leads to the formation of unstable heterogenotes (diploid for the gal locus), such heterogenotes yielding very high efficiency lysates on induction. There is no comparable heterogenote formed by transducing phages in general transduction.

4. Episomes. This final section deals with those agents capable of transferring heritable traits from cell to cell by infection-prophage, fertility factors, colicinogenic factors and resistance transfer agents. An episome possesses certain characteristic features (i) it can be added to or lost from the bacterial genome and is a non-essential genetic determinant (ii) if present in a bacterium, it can exist in two sites, cytoplasmically - in which case it replicates faster than the bacterial genome - or integrated - with a specific site of attachment to the bacterial genome (iii) each episome is transferred by a specific agent. (Jacob, Schaeffer and Wollman, 1960).

(a) Lysogenic conversion. When a temperate phage is present in its integrated state, i.e. as prophage, it confers on the host cell important new properties, the most significant of which is that it confers immunity to superinfection with homologous phages. The mechanism that prevents vegetative multiplication and lysis is an immunity repressor (Jacob, Schaeffer and Wollman, 1960).

This is an excellent example of the regulator mechanisms present in phage, since all the functions directly involved in the completion of the lytic cycle are effectively repressed, presumably by blockage of a common operator gene. Nevertheless, certain phage genes are apparently capable of functioning regardless of the presence of the repressor system which blocks phage maturation. The phage genes referred to are the genes controlling somatic antigen synthesis in certain salmonellas. In some species, the presence of a particular somatic antigen depends on the parent cell's lysogenisation by phage. For example, Zinder (1957) showed that the initiation of somatic antigen 1 synthesis came with infection by phage P22. The phages ε^{15} and ε^{34} control the synthesis of antigens 15 and 34 in salmonellas of group E (Uetake and Hagiwara, 1961). The synthesis of these somatic antigens is non-essential to both the phage and the bacterium, since the host-bacteriophage equilibrium is not disturbed when these antigens are lost. Lysogenisation (or phage conversion) differs from transduction in that the phages responsible are not defective, and every one is active in its ability to affect host properties.

Two other groups of episomes, the fertility factors and colicinogenic factors, bestow on cells carrying them the ability to mate and donate bacterial chromosomal characters.

(b) The fertility factor F in E.coli controls the

"sex" of the cells by its presence or absence. Those cells carrying F in a cytoplasmic state are F+ or male, those in which F is absent are F- or female. Mating occurs between F+ and F- cells but also to a slight degree between two F+ cells. When the F factor is integrated on the chromosome of the carrier cell, the cell is referred to as an Hfr male. The presence of the fertility factor results in changes in the surface properties of the cell, thought to be due to the production of a conjugation apparatus (Ørskov and Ørskov, 1960, Sneath and Lederberg, 1961).

(c) The production of the protein antibiotic substances called colicins, produced by many of the enterobacteria, is controlled by the presence of the colicinogenic factors. As well as bestowing on carrier cells this ability to produce colicins lethal to other cells, colicinogenic factors also act as vectors of genetic material during cell-to-cell contact. This has been well studied in Salmonella, where the phenomenon of colicinogeny has been successfully applied to the study of the chromosome (see Smith and Stocker, 1962). In many ways, the colicinogenic factors resemble prophages, since both are potentially lethal agents, both give immunity to cells carrying them and both can be induced to replicate autonomously, which results in lethal effects on the carrier cell. Fredericq (1963) suggests that colicinogenic factors are virulent bacteriophages, so defective that they can no longer lyse

the cell, but with the gene for lethal protein synthesis still present. Of course, there is an apparent relationship between colicinogenic factors and the F agent in the way in which both can initiate a conjugation process. The differences between the three types of episome are obviously not great, and Fredericq considers all three to be very closely related.

(d) From humans in Japan, large numbers of Shigellas were isolated with high resistance to chloramphenicol, the tetracyclines and streptomycin. Investigation revealed that this antibiotic resistance was brought about by a class of episomes called resistance transfer agents. (Nakaya, Nakamura and Murata, 1960). These factors are transferred at a high rate from Shigella to Shigella during cell-to-cell contact, and further studies revealed that this transfer occurred between Shigella and a wide range of susceptible genera - Salmonella, Arizona, Escherichia, Citrobacter, Klebsiella, Enterobacter, Hafnia and Proteus. Such transfer, since it is complete within minutes, offers a significant means of altering the hereditary characters of a large number of bacteria. These transfer agents, presumably, produce a specific antigen at the cell surface like the prophage, fertility and colicinogenic factors.

The ability of episomes to alternate between an autonomous and an integrated state is important, since it often leads to the incorporation of fragments of the donor

genome (Jacob, Schaeffer and Wollman, 1960), and when this happens, the bacterial gene is transferred to the recipient cell as part of the episome. Such a gene can be integrated into the recipient cell by recombination. When the transferring episome is a sex factor, the process is called F-duction, when a phage, -transduction. From this review, it would seem that the differences between the different episomes and the processes they control are not too great. No doubt, intermediate forms, still classifiable as episomes, will be discovered. All the episomes mentioned above can be rapidly transferred from cell to cell by direct contact, except phage, which does not require the interaction of two cell types for its transmission. Other means of transmission are also available; when in the integrated state, all can be transferred during conjugation as part of the donor genome, and all can be transmitted by transduction.

Complex loci in bacteria.

Demerec (1956) and his co-workers (1956, 1955, 1958) collected a large number of independently isolated, mostly natural, mutants of S.typhimurium defective in the synthesis of amino acids, purines and galactose. When pairs of these mutants, defective in any single character, were tested in pairs (by means of transduction), it was found that recombination and complementation occurred among certain of the mutants. The transductional analyses divided the mutants into groups which closely agreed with

the grouping arrived at from biochemical data, such as feeding experiments and the accumulation of intermediates. A group of non-complementing mutants represent mutations which all lie within a restricted segment of the chromosome, the gene locus (Demerec, 1956) or the cistron (Benzer, 1955), which is the unit of function. Within this locus, however, both recombination and mutation are possible. Each locus is subdivided into smaller segments called sites, and it is the changes in these sites which represent the many alleles presented by a locus. These sites, although they represent the limits of recombination, do not, of course, represent the limit of mutation which can be a single base pair altered on the chromosome. Mutants were discovered which overlapped cumulatively the single sites of many other mutants, some deletion mutants extending over several loci. This detailed analysis by Demerec and his co-workers was the first demonstration of a phenomenon well-known in higher organisms, viz., non-identical allelism or complex structure of loci. This was simultaneously discovered in phage (Benzer, 1955). The results of Demerec et al (1955) showed that complex loci were not peculiar to any particular locus, but to all which have been studied.

A similar detailed study by Hartman and his co-workers (1960a,b) revealed that the synthesis of histidine was controlled by eight distinct loci, six of which were identifiable with definite steps in the synthesis, while the group at each end was, at that time, of unknown function.

The work of both schools had revealed that the gene loci controlling the synthesis of the different enzymes involved sequentially in a particular metabolic pathway were closely linked (Demerec and Hartman, 1959). Close linkage of allied gene loci controlling antigenicity, morphology and formation of flagella has already been mentioned. Various speculations were presented to explain these findings. For example, Pontecorvo (1956) had found that the gene loci controlling tryptophane synthesis in Neurospora, although near, were not clustered as they are in Salmonella, and suggested that this was because in lower organisms such as bacteria, the metabolic functions are still performed by the nucleus, whereas, in Aspergillus and Neurospora, they have been delegated to the cytoplasm. It has since been established from the excellent work of Jacob and Monod(1961) that simultaneous induction or repression is the rule in these metabolic sequences. The genes clustered in this way are probably all controlled by a single "operator" (switch) gene at the end of the sequence. This operator gene is thus the unit of co-ordinated action, but, in the presence of a specific repressor, produced by a "regulator" gene, the operator gene is blocked and the whole system is switched off. This clustering of genes can, therefore, be readily understood, since it allows efficient and speedy regulation of their activity which is important in the conservation of metabolites and energy. The genes of unknown function at the end of the histidine loci (Hartman

et al, 1960a,b) are now believed to be such operator genes.

Reports in the last few years make it seem likely that many of the mechanisms referred to in the above resume have been influential in the modification of bacterial genotype. The demonstration of "in vivo" recombination between Escherichia and Salmonella (Schneider et al, 1961) and the increasing reports of types intermediate to the classical genera are examples which come readily to mind as of possible evolutionary significance. Intergeneric fertility has been reported repeatedly: between E.coli and Shigella (Luria and Burrows, 1957), between E.coli and Salmonella (Baron, Carey and Spilman, 1959., Brinton and Baron, 1960, Ørskov, Ørskov and Kauffmann, 1961), between Salmonella and Serratia marcescens (Falkow, Marmur, Carey, Spilman and Baron, 1961). Most of these observations of intergeneric fertility were explained by episomal infection, not by sexual recombination. The former takes place among enterobacteria with significantly differing DNA base compositions, although conjugation requires organisms of similar DNA base composition (Marmur et al, 1961). This was further proof of the speed and efficiency of episomic infection in natural conditions. It is hoped that this review has drawn attention to the many different mechanisms which bacteria have available for the monification of their genotype, many of them being unique to micro-organisms.

With all these possibilities in mind, it was

decided to investigate the control of fimbriation in Salmonella among a large number of non-fimbriate mutants of S.typhimurium in which we might determine which of our phenotypically similar mutants were allelic and a proof of the complex nature of the fimbriation locus. This was done by means of the reciprocal transduction technique successfully used by Demerec and Hartman (1959) in their studies, because of the ease of handling large numbers of cultures for transduction work as compared to other methods of genetic transfer. The ease with which the active DNA (i.e. transducing lysates) could be prepared was an additional recommendation.

MATERIALS AND METHODS

Table 1. Source, date and characters of fimbriate strains of *Salmonella typhimurium*.

Sa reference no.	Supplier and Supplier's No.	Source	Date	Place	Colindale phage types		Other characters.
					Old	New	
206	H.A.Wright	Man	1955	Scotland	1a	2	inos.-ve FIRP
375	B.A.D.Stocker	Boyd's Q_1 indicator strain * Sa 375 carrying P 22 (Alb) Lilleengen phage-type strain LT-2 ≠			1	1	str.- r.
736	L.O.Kallings.	LT-5			1b	4	Man/Sweden
740		LT-6A			1a	2	
741		LT-6B			1a	2	
742		LT-6BL			1a	2	
744		LT-8			1a	2	
745		LT-9			1a	u57	col. +ve.
746		LT-10			Ivar5	u-	
747		LT-11			1	1	
748		LT-12			2b	16	
755		LT-19			2b	N.T.I.	
848	J.Taylor./3496/60 S.R.L.	Pork	1960	Holland	u	u	poorly fimbriate
1302	B.A.D. Stocker 2M/358	Bacon	~~1962~~	England	Ivar2	u41	
1304	from E.R.L. 2M/372	Man	"	Wales	2	9	
1404	2M/11	Guinea-pig	1961	England	Ivar5	u9	inos.-ve FIRP
1451	2M/237	Cattle	1962	"	1b	4	poorly fimbriate
1566a	P.R.Edwards 2495/62	Pig	1962	U.S.A.	1a	u57	col. +ve.
1179	G.N.Cooper. 392465	Man	1962	Australia			
1180	" 408976	"	"	"			
1541	P.R.Edwards 6501/61	Man	1961	U.S.A.	u	u	
6768	E.R.L.	Man	1958/59	England	u	u	ex Brighton
6899	"	"	"	"	u	u58	"

S.R.L. = Salmonella Reference Laboratory, Colindale, London.
E.P.L. = Central Enteric Reference Laboratory, Colindale, London.

* see Boyd and Bidwell (1957)
≠ see Lilleengen (1948)

Sa 1179, 1180, 1541, 6768 and 6899 are fimbriate, rhamnose non-fermenting strains.

Organisms.

Escherichia coli: E.coli 23 was obtained from Dr. Joan Taylor, Salmonella Reference Laboratory, Colindale, London. This strain was fimbriate, non-flagellate and non-capsulate. E.coli AI22 was obtained from Professor J.P. Duguid; it has been described previously, Duguid et al, 1955.

Shigella flexneri: Sh.flexneri strain FIaI was obtained from Professor J.P. Duguid. This strain is N.C.T.C. 8192. It has been described previously, Duguid and Gillies, 1957.

Salmonella paratyphi B: six strains were obtained from Professor J.P. Duguid; these were Sa 66, 964, 1309, 1313, 1361 and 1363. These are fimbriate, but non-haemagglutinating, otherwise typical, S.paratyphi B strains.

Salmonella typhimurium: all S.typhimurium strains used in this investigation were obtained from Professor J.P. Duguid from various sources. The source and characters of the fimbriate S.typhimurium strains are outlined in table I. The source and characters of the non-fimbriate strains, classified as FIRN and FIRN', are shown in table 2. Other non-fimbriate strains will be discussed in the text (section I of experimental and results).

Nutrient broth was prepared from Oxoid dehydrated broth, adjusted to pH 7.0; nutrient broth (A) contained Oxoid bacteriological peptone, 10g, Oxoid lab lemco, 10g., and NaCl, 5g., per 1000 ml. distilled water. This was adjusted to pH 8.0, steamed to precipitate phosphates, cooled overnight, filtered and adjusted to pH 7.0. Graduated volumes were dispensed in metal-stoppered test-tubes and autoclaved;

Table 2. Source, date and characters of non-fimbriate *Salmonella typhimurium* strains called FIRN*

Sa Ref. No.	Supplier and Supplier's No.		Source	Date	Place	Colindale phage types		other characters.
						Old	New	
	Stocker	SW573	Man	1952	U.S.A.	2a	13	fla- mot-
	original	SW576				2a	13	fla+ mot+
	from	SW577						curly fla, phase 1
	Friewer and	SW578				2a	13	fla+ mot-
	Leifson,	SW580	N.C.T.C. 8298					"
100	and	SL43						"
	mutants.	SL272						fla+ mot +
	Meynell	SL43S	ex SL43					
	mutants.	SW582	ex SW578					
		SW583	ex SW580					
6683	B.A.D.Stocker	M6683	Fowl	1959	S.Rhodesia	un	ul7	col 1, cys-
6758	from E.R.L.	M6758	"	"	England	2c	14	col 1.
6759		M6759	"	"	"	2c	14	col 1.
6760		M6760	"	"	"	2c	14	col 1.
6822		M6822	Man	"	"	2c	14	col E_1.
6825		M6825	"	"	"	2c	14	col 1.
7471		M7471	?	"	"	L	I	non-lysogenic
528	J.Taylor.	2579	Duck	1960	England	2a	13	
546	from S.R.L.	2690	Man	"	"	2c	14	
549		2790	"	"	"	2b	14a	
805		2952	"	"	"	2c	14	
807		3002	"	"	"			
808		3003	"	"	"			
809		3015	"	"	"	2c	14	
816		3107	"	"	"			
834		3318	Food	"	"			
845		3432	Man	"	"			
850		3105	"	"	"	2a	13	O-antigen 5-ve
855		3200	"	"	"	2c	14	"
856		3398	Turkey	"	"			"
602	J.E.Wilson.		Fowl	1955	Scotland	2c	14	
603	Lasswade		Turkey	"	"	2c	14	
604			Fowl	"	"	2c	14	
605			Turkey	"	"			
607			Fowl	"	"	2c	14	Harhoff 15.
608			"	"	"			
609			"	"	"	2c	14	
610			Turkey	"	"	Ivar2	u41	
611			Sparrow	1960	"	2c	14	
612			Turkey	1955	"			
613			"	"	"	2b	14a	col.1.
614			"	"	"			
616			"	"	"	2c	14	
618			"	"	"	2c	14	
620			Fowl	"	"			
621			"	"	"			
622			"	"	"			
623			"	"	"			
625			"	"	"	2c	14	

Table 2 (continued)

Sa Ref. No.	Supplier and Supplier's No.		Source	Date	Place	Colindale phage types		other characters.
						Old	New	
626	J.E.Wilson.	IPH7/ 4487	Fowl		Scotland			
628	Lasswade	509	"		"			
629		IPH8/978	"		"			
630			"		"			
631		363	Egg		"	2c	14	
632		84	"		"			
633		IPH8/815	Fowl		"			
634		R.I.R.7222	"		"			Harhoff 16
635		315	"		"	2a	13	Non-motile
636		2205	"		"	2c	14	Harhoff 17
639		BrL506	"		"			Harhoff 15
701	L.O.Kallings	112.59	Cattle	1959	Sweden	2a	13	Lilleengen phage-type 4
702		113.59	Gull	"	"	2a	13	Lill.4.
703		114.59	Turkey	"	"	2a	13	"
704		132.59	Parrot	"	"	u	ul7	Lill.un.
705		142/159	Man	"	"	2c	14	"
706		160.59	Guinea-pig	"	"	2c	u45	Lill.4.
707		3.60	Turkey	1960	"	2c	14	Lill.un.
708		5.60	Cattle	"	"	2c	14	"
709		6.60	Pig	"	"	2a	13	Lill.4.
710		9.60	Man	"	"	2c	14	Lill.un.
711		24.60	"	"	"	2c	14	"
713		28.60	"	"	"	2a	13	Lill. 3.
714		32.60	"	"	"	2c	14	Lill.un.
715		50.60	Guinea-pig	"	"	2c	14	"
716		59.60	"	"	"	2c	14	"
717		61.60	Man	"	"	2c	14	"
718		72.60	Guinea-pig	"	"	2c	14	"
719		126.60	Cattle	"	"	2c	14	"
720		134.60	Man	"	"	2a	13	Lill. 13.
721		154.60	"	"	"	2c	14	Lill.un.
722		158.60	Pig	"	"	2c	14	"
733	S.Tulinius.	11338		1955	Denmark	2c	14	
734	"	18466		1959	"	1	1	
735	L.O.Kallings.	LT-L		1960	Sweden	1	1	Lill.1.
738		LT-4		"	"	2a	13	Lill.41
760		LT-23		"	"	un	29	Lill.23.
932	P.R.Edwards.	553	Turkey	1961	U.S.A.	un	29	
645	W.J.Sojka.	SI691	Cattle	pre 1960	England	2c		Harhoff
652		SI420	Pig	"	"		unl7	" 16
653		SI281	Mink	"	"	2c		" 17
654		SI404	Chinchilla	"	"		14a	" 15, col.1.
563	J.P.Duguid.		Man	1960	Scotland	2c	14	
575	H.A.Wright.		"	"	"	2a	13	
581	"		"	"	"			
582	"		"	"	"	I	I	

Table 2 (continued).

Sa Ref. No.	Supplier and Supplier's No.		Source	Date	Place	Colindale phage types		Other characters.
						Old	New	
598	J.E.Phillips.	2534	Dog	1952	Scotland	2c	14	
874	R.R.Gillies.	27960	Man	1961	"			col I.
1366	"	55592	"	1962	"	2c	14	
1376			"	"	"	Ivar2	un41	auxotroph.
1516	G.N.Cooper.		"	"	Australia	2c	14	
								ex:
1285	B.A.D.Stocker	E.R.L.2M2	Abattoir	1961	England	2c	14	Ipswich
1286	from E.R.L.	2M4	"	"	"	2c	14	"
1287		2M55	Man	"	"	2a	13	Belfast
1288		2M57	"	1962	"	2c	14	Dorchester
1289		2M65	"	"	"	Ivar2	u41	Newport
1290		2M67	Chick	"	"	2a	13	Lasswade
1292		2M102	"	"	"	2c	14	Leeds, fla-
1293		2M113	Man	"	"	2c	14	Stafford
1294		2M183	"	"	"	2b	14a	Nottingham
1296		2M195	Gull	"	"	2a	13	Lasswade
1297		2M200	Man	"	"	2c	14	Lincoln
1298		2M242	"	"	"	I	I	Watford
1300		2M342	Egg	"	"	I	I	Ipswich
1301		2M356	Duck	"	"	2c	14	Lincoln
1303		2M364	Pig	"	"	2c	14	Preston
1701	T.Anderson.	M1939		1948	"	2c	14	Conway
1702	from E.R.L.	2146		1949	"	2c	14	Oldham
1703		2246		"	"	2c	14	Preston
1704		2358		1950	"	2c	14	Cambridge
1714		IM2104		1961	"	2c	14	Lincoln fla-
1720		2M1994		1962	"	2c	14	Nottingham, fla-
1725		3M 253		1963	"	2c	14b	Liverpool
1729		IM5364		1961	"	2b	14a	Stafford
1569	P.R.Edwards.	2592	Man	1962	U.S.A.	2a	13	col X.
1576		2792	Chick	"	"	2a	13	col X.Pennsylvania
1577		2798	Man	"	"	2a	13	col X.
1583		3008	Chick	"	"	2a	13	col X. Maine
1585		3061	Man	"	"	un	un	col X.
6624	B.Stocker.		Man	1958	England	2c	14	Oxford
6629	from E.R.L.		"	"	"	2a	13	Guildford
6724			"	"	"	2c	14	Eastbourne
6785			"	"	"	2c	14	Epsom
6788			Egg	"	"	2c	14	"
6799			Toucan	"	"	un	ul7	Bristol
6907			Man	"	"	2c	14	
6908			Man	"	"	2c	14	Edmonton
6925			Dog	"	Scotland	2a	13	inos+= FIRN'
6929			Egg	"	England	2c	14	Hull
1642	J.E.Wilson.	P1693/E	Chick	1962	"	2a	13	
1643	Lasswade	28/62	Sparrow	"	Scotland	2a	13	
1644			"	1960	"	2c		
1645		961	Pigeon	1962	England	2c	14	

* These FIRN strains were collected by Professor J.P. Duguid. All FIRN strains are non-fimbriate, inositol non-fermenting and rhamnose non-fermenting. All strains are also flagellate except (certain) Sa 100, 635, 1292, 1645, 1714 and 1720.

nutrient broth (B) contained Oxoid bacteriological peptone, 10 g., Oxoid yeast extract, 5g., and NaCl, 5g., per 1000 ml. distilled water, adjusted to pH 6.0.

Peptone water sugars contained Oxoid bacteriological peptone 10 g., and NaCl, 5g., per 1000 ml. distilled water. After autoclaving, 10g. of the required sugar (from a sterile 10% w/v solution) and 14 ml. of Andrade's indicator were added. 5 ml. amounts were dispensed in test-tubes containing Durham tubes, and tyndallised. The sugars and sugar alcohols most commonly used were trehalose, xylose, rhamnose, mannitol and inositol.

Phosphate broth. Equal amounts of double-strength nutrient broth (A) and 2% Na-K phosphate buffer, both pH 7.0, were mixed just before use, tubed and steamed for 90 min. The phosphate buffer contained KH_2PO_4, 0.7 g., and Na_2HPO_4, 1.3g., per 100 ml. distilled water.

Citrate and tartrate media contained Oxoid bacteriological peptone 10g., and 24 ml. of a 1:500 solution of bromothymol blue per 1000 ml. distilled water. This was adjusted to pH 7.4 and autoclaved. Before use sterile 10% sodium citrate or 10% D-sodium tartrate were added. This was tubed and steamed for 90 min.

Stern's glycerol fuchsin medium was made from three solutions. Solution 1: meat extract, 10g., peptone, 20g., per 1000 ml. distilled water, adjusted to pH 8.0. Solution 2: saturated alcoholic solution of basic fuchsin. Solution 3: fresh 10% (w/v) anhydrous sodium sulphite. These were mixed as 100 ml. solution 1, 0.2 ml. solution 2, 1.66 ml. solution 3

and 1 ml. glycerol added. This final mixture was tubed and autoclaved (Edwards and Ewing, 1955).

Nutrient agar. Nutrient broth was solidified by the addition of 12g. of Davis agar, type P, per litre of broth.

Phosphate agar. To 400 ml. of nutrient broth (A), 5g. of Oxoid Ion agar were added and steamed for 90 min. 100 ml. of sterile 5% Na-K phosphate buffer, pH 7.0, were added to give finally 1% phosphate agar.

Minimal agar (DM) contained K_2HPO_4, 7g., KH_2PO_4, 3g., $(NH_4)_2 SO_4$ 1g., $MgSO_4$, 0.1g., and washed Davis P agar, 20g., per litre of distilled water. This was sterilised by autoclaving and sufficient sterile 10% (w/v) solution of the required sugar added just before pouring to give a final concentration of 0.3%. Occasionally, this was enriched by the addition of 10 ml. nutrient broth per litre.

Eosin-methylene blue agar (EMB) contained Oxoid bactopeptone, 3g., K_2HPO_4, 0.6 g., eosin yellow, 0.4g., methylene blue, 0.065g., and washed agar, 20g., per litre of distilled water. The pH was 6.8 and the final concentration of sugar was 0.5%.

Haemagglutination tests.

These were made with 3% guinea-pig or horse cells in saline suspension for MS adhesins (Duguid et al, 1955).

To detect the presence of fimbriae, drops of bacterial suspension and red cells were mixed in the depressions of a porcelain tile, which was mechanically shaken. Clumping of red cells was normally seen quickly when loopfuls of deposit were tested. The mannose sensitivity of the reaction was

detected by the addition of 2% (w/v) mannose solution which inhibits the MS system (Duguid and Gillies, 1957).

The haemagglutinating power (HP) of the cultures was detected by the method of Duguid and Gillies (1957).

Doubling dilutions of free fimbriae were made in 0.5 ml. amounts of diluent, 0.75 M $MgCl_2$. 6 H_2O, in Wassermann tubes. Equal volumes of 1% guinea-pig or horse cells were added, shaken and the tubes left overnight at 4° before reading. The last tube showing a definite ++ haemagglutination was the end-point. Haemagglutination of the free fimbriae appeared as a diffuse agglutination of red cells on the bottom of the tube.

Transduction procedures.

(i) The phages used to prepare lysates were both A-type phages, Boyd and Bidwell (1957), viz. Alb, P22 as used by Zinder and Lederberg (1952) and, in special cases A2c. The propagating strains carrying these phages were SL 377 and SL 382, respectively (supplied by Dr. B.A.D. Stocker).

(ii) Preparation of lysates. Phage lysates were prepared by the soft-agar technique of Swanstrom and Adams (1951). When fimbriate strains were used these were taken through two 48hr. nutrient broth subcultures to allow phenotypic expression of the fimbriation gene. To 20 ml. amounts of molten semi-solid nutrient agar (incorporating 0.3% w/v Davis Agar) in sterile 1 oz. vials, 0.5 ml. of the donor strain from an overnight broth culture and 0.5 ml. of a previously calculated test dilution of seed phage were added, mixed and

5 ml. amounts layered on to thick nutrient agar plates. Semi-confluent lysis appeared at different times from 4 to 10 hr. The phage from such plates was recovered by homogenisation of the overlays in 5 ml. amounts of broth and clarification by centrifugation at 5,000 G for 30 min. The phage-containing supernatant was heat-sterilised in a 56° water-bath for 60 min. Phage lysates were stored at 4° for months without loss of activity.

(iii) Titration of phage. Phage lysates were counted by the Miles and Misra (1938) method. 0.02 ml. volumes of serial ten-fold dilutions of phage in nutrient broth were spotted onto a dried lawn of indicator strain SL 375, Boyd's Q1 universal indicator strain, and allowed to dry. After overnight incubation at 37°, the number of plaques in such drops were counted and the number of plaque-forming particles per ml. calculated.

(iv) Transduction to fimbriation.

Eighteen-hour nutrient broth cultures of the recipient test strain were inoculated into nutrient broths, and incubated at 37° until the cell concentration was approximately 2×10^8 per ml., (usually 3 - 3½ hr.). After addition of the phage at multiplicities from 1 - 3, the phage-bacteria mixtures were gently shaken and, thereafter, incubated undisturbed for 48 hr. to allow pellicle formation of fimbriate transductants to occur. Subcultures were made from these into fresh broths, which were incubated for a further 48 hr. The deposits from the centrifuged first 48 hr. subcultures were tested for haemagglutination.

(v) Isolation of fimbriate lines.

When thedeposit from such an experiment was found to be haemagglutinating, a loopful of it was streaked on nutrient agar. After 24 hr. at 37°, single colonies were picked into individual broths and pure fimbriate lines isolated in static aerobic nutrient broths after 48 hr. incubation at 37°.

(vi) Characterisation of isolated lines.

Salmonella typhimurium fimbriate transductants from intra-species transduction were identified by their sugar spectra which differentiates FIRP donor and FIRN recipient strains. Other serotypes were identified by slide agglutination tests with salmonella sera obtained from The Standards Laboratory, Colindale, and, where necessary, by tube agglutination (Mackie & McCartney, 1960). Pure fimbriate lines were checked for the presence of fimbriae by (a) the tile test, (b) electron microscopy.

(vii) Transduction to flagellation.

The procedure was initially as in (iv). After the addition of phage, 10 min. were allowed for adsorption at 37°. The phage-bacteria mixture was spun, and loopfuls of the deposit spotted on the central point of the medium used by Stocker et al (1953). This contained 8% (w/v) gelatin and 0.4% (w/v) agar in a nutrient broth base. Overnight incubation at 37°, allowed flagellate transductants to be picked from the periphery of the plate, to which points they had migrated. These were tested by hanging-drops on loopfuls from 6 hr. nutrient broths.

(viii) Transduction to Prototrophy.

The procedure was as in (vii). In this case, the whole deposit or dilutions were pipetted onto plates of minimal medium, supplemented with the required nutrients, and spread until dry with a glass rod bent at 90°. Transductant colonies were purified, after 72 hr. at 37°, by restreaking on to fresh minimal media.

Bacterial counts.

These were made by the method of Miles and Misra(1938).
(a) 0.02 ml. volumes of serial tenfold dilutions were spotted on to dried nutrient agar plates and allowed to dry.
(b) 0.1 ml. volumes from such dilutions were spread on plates. Broth and saline were the diluents used.

Growth of suitably diluted broth or saline suspensions was determined by the absorption at 530 mμ on a Unicam spectrophotometer, using 10 mm. glass cuvettes.

Electron microscopy.

(i) Preparations for electron microscopy were fixed with 0.25% formaldehyde and shadowed with gold-palladium alloy. The preparations were viewed on a Metropolitan-Vickers EM6 Model.

(ii) Preparations were also made by the gel-filtration method of Kellenberger and Kellenberger (1954). The agar for filtration contained Oxoid tryptone, 7.5 g., per 450 ml. double-distilled water. This was autoclaved for 20 min., and 20 plates poured. The plates were dried at 60° for 60 min., then flooded with petrol ether, which was removed

by evaporation. A 0.2% (w/v) solution of collodion (nitrocellulose) in amyl-acetate was poured on the surface of these plates, immediately removed and the plates dried inverted for 6 hr. Preparations spread over the surface of these collodion films filtered quickly. If the preparation was in distilled water, one drop of sterile tryptone was added to aid spreading. The preparations were then fixed in an atmosphere of formaldehyde vapour for 7 min. and sections 1 cm. square cut from the agar. The collodion film was floated on a 2% (w/v) solution of lanthanum nitrate and mounted on grids. These were dried on blotting paper and shadowed.

Antisera.

Adult rabbits were injected with bacteria, (fixed in formaldehyde, and suspended in saline to a concentration of 10^8 organisms/ml.) by two subcutaneous injections (o.25 and 0.5 ml.) and four intravenous injections (0.25, 0.5, 0.5 and 1.0 ml) at three-day intervals. Sera were collected 11 days after the final injection. Fimbriae (containing 1mg. protein per ml.) were injected according to the method of Kerridge et al (1962) for detached flagella: i.e. a total volume of 7 ml. in 5 intravenous doses over a period of four weeks. Control preimmunisation sera were always taken. Sera were preserved by the addition of sodium azide to 0.02% (w/v).

Gel diffusion.

This was performed by the standard methods (Ouchterlony, 1948, and Crumpton and Davies, 1956). The agar for diffusion contained Oxoid No.2. Ion agar 1 g., and

sodium azide, 0.02 g., per 100 ml. of 0.01 M Na-K phosphate buffer, pH 7.0. Ten ml. amounts were poured in plastic disposable petri dishes to a thickness of 3 mm. Holes for antigen and antibody were cut from the agar by a Feinberg aluminium agar-gel cutter, giving a concentric 7-well pattern (Cat. No.1801, Shandon). Antigen solutions in phosphate buffer and antisera were placed in the holes with sterile pasteur-pipettes. The plates were left at room temperature in a humid atmosphere for 14 days to allow full development of precipitation lines. Squares (2" x 2") were then cut from the fourteen-day test agars, and washed repeatedly in physiological saline for two days followed by two days of repeated washing in distilled water. The washed squares were then allowed to dry overnight at room temperature on Kodak lantern slide cover glasses ($3\frac{1}{4}$" x $3\frac{1}{4}$").

Staining.

The preparations were then stained by a modification of the method of Rondle and Carman (1956). The slides were immersed for 5 min. in a 1% (w/v) solution of naphthalene black in a mixture of methanol, distilled water and acetic acid (5:4:1,v/v) and then decolourised with the above solvent for five minutes. They were dried at room temperature. This procedure stains the precipitation lines blue and leaves the agar matrix unstained.

Adsoprtion of phage.

Test strains were grown in 10 ml. aerated broths for 4 - 5 hr. until they had grown to a density of approximately 5×10^9 per ml. Dilutions pf these cultures

were made in broth as diluent and 0.1 ml. amounts at appropriate dilutions counted by the pour-plate method. Immediately the test strains had been counted, 4.5 ml. amounts of each were placed in small test tubes and 0.5 ml. of P22 phage lysate added at known multiplicities. After mixing, these were incubated at 37° for 10 min., when the mixture was centrifuged in a cold room at 8,000 G for 12 min. to remove bacteria plus adsorbed phage. Small volumes of the supernatant were removed and diluted. Phage was assayed in this by plating 0.1 ml. amounts of known dilutions, plus indicator strain, by the agar layer method, (Adams, 1950).

Treatment with mutagens.

(i) Manganous Chloride.

Overnight broth cultures of non-fimbriate strains were centrifuged and resuspended in 5 ml. amounts of pre-warmed 0.04% (w/v) manganous chloride (Hartman, 1956). These were incubated for one hour at 37°. The treated cells were then centrifuged, the manganous chloride removed and replaced by broth. These were incubated statically through two forty-eight hour nutrient broth subcultures, and the deposits tested for haemagglutination.

(ii) Ultraviolet Irradiation.

Overnight broth cultures of non-fimbriate strain s were centrifuged, washed in 0.15 M NaCl, centrifuged again and resuspended in 10 ml. liquid (DM) minimal medium. These suspensions, approximately 1 x 10^9 cells per ml., were irradiated in Petri dishes with an ultraviolet lamp. Survivors were of the order of 10%, and 1 ml. amounts were

pipetted into nutrient broth. These were tested for haem-agglutination after two forty-eight hr. subcultures.

(iii) Ethyl methane sulphonate.

Overnight broth cultures of non-fimbriate strains were centrifuged, washed in 0.15 M NaCl, centrifuged again and the deposit resuspended in 3 ml. 0.4M ethyl methane sulphonate for 15 min. at 37°. The suspension was then diluted by the addition of 7 ml. of 0.02 M Na-K phosphate buffer, pH 7.0, centrifuged and resuspended in nutrient broth. These were subcultured through two forty-eight hour broth subcultures.

Preparation of fimbriae.

Strongly fimbriate cultures of E.coli 23, S.typhi-murium 635 fim +, or Shigella flexneri FIaI were inoculated into Roux bottles containing 150 ml. amounts of nutrient broth B. These were incubated on their side for 48 hr. at 37°. Harvesting and defimbriation will be discussed in detail in the text of section III of the experimental and results.

Purification of fimbriae.

The crude fimbrial preparations isolated were equilibrated by dialysis for 24 hr. against the appropriate buffers, and applied to ion-exchange columns, previously equilibrated for 24 hr. against the same buffer. The columns were either diethylaminoethyl - (DEAE) - or carboxymethyl - (CM) celluloses, respectively anion and cation exchangers. After application of the crude fimbriae,

fractions were eluted with a salt gradient at a rate of 12.5 ml/hr./cm^2. surface area. Fractions (6.25 ml.) were collected at 30 min. intervals, automatically timed by a Locarte fraction collector. The salt gradient was achieved by pumping from a reservoir of 2 M sodium chloride in phosphate buffer into a mixing vessel containing 200 ml. of the original buffer, which was stirred continuously by a magnetic stirring device. The fractions were tested for protein and fimbriae, and all fractions showing haemagglutinating activity pooled and concentrated by dialysis against polyethylene glycol and distilled water.

Chemical methods.

Nitrogen. Fimbriae in duplicate samples were digested by a micro-Kjeldahl technique and, after distillation in a modified Markham still, nitrogen was titrated as ammonia against 0.01 N H_2SO_4 with methyl red-methylene blue indicator.

Phosphorus was estimated by the Fiske and Subba Row method (1925).

Dry weight of fimbriae in distilled water was estimated by drying duplicate 2 ml. samples to constant weight in an oven at 100° for 4 hr.

pH was measured on a Pye Dynacap meter.

Cabbohydrate. Reducing sugars (estimated as glucose) were calculated by the modified anthrone method (Fairbairn 1953).

Protein and nucleic acid were measured by the respective absorptions at 280 mμ and 260 mμ on a Unicam spectrophotometer.

Calculations were made by reference to a standard nomograph, based on the extinction coefficients of enolase and nucleic acid.

Chromatography. Fimbriae were hydrolysed in 6 N HCl in sealed ampoules for 24 hr. at 100° in a boiling water bath. HCl was removed by evaporation, the residue resuspended in distilled water and the process repeated three times. Amino acids were detected by paper chromatography on Whatman No.1. paper. The chromatograms were descending: the first dimension was irrigated for 24 hr. in n-butanol+acetic acid+ water (4:1:5. v/v) and the second dimension irrigated in phenol + water (80 + 20) containing 0.3% (v/v) ammonia. After drying, the chromatograms were sprayed with 0.2% (w/v) ninhydrin in 95% (v/v) ethanol, and spots allowed to develop at room temperature for 6 - 18 hr.

Competition Experiments.

The media used were 10 ml. amounts of nutrient broth (A) or phosphate broth in cotton-wool stoppered test-tubes, and 20 ml. amounts of nutrient or phosphate agar in plastic disposable dishes. 40 ml. amounts of broth in 500 ml. conical flasks were shaken on a reciprocating shaker. Rotated cultures contained 40 ml. of broth per litre bottle, rotated automatically at a speed of 12 revolutions per minute. Micro-aerophilic conditions were achieved using a McIntosh and Fildes' jar, of capacity 1 litre, in which 920 c.c. of the air was replaced by hydrogen. Cultures were incubated anaerobically in a McIntosh and Fildes' jar in an atmosphere of hydrogen.

The strains used were all S.typhimurium.

(i) naturally isolated FIRP and FIRN strains

(ii)double transductant of a FIRN, rha+ fim+, v. parent rha- fim- FIRN.

(iii)fimbriate transductant of a FIRN, also rha + by selection of a mutant, v. parent rha- fim- FIRN.

All strains used in competition were tested to show that no reciprocal lethal effects were produced by (a) phage (b) colicines.

The challenger and challenged strains for competition were subcultured from Dorset egg bijoux on to nutrient agar for 24 hr. Single colony lines were picked on to Dorset egg slopes and these isolated lines always subsequently used for comparative work. After subculture on agar, single colonies were picked into broth for 24 hr. (exactly) and, after homogenization of the 24 hr. broth culture, bulging loopfuls subcultured into fresh broths. These cultures grew in 3 - 3½ hr. to an approximate cell concentration of 3×10^8 cells per ml.

The challenger broth was then homogenized and serial tenfold dilutions made in 4.5 ml. amounts of broth. Single 0.02 ml. drops of the challenger from calibrated pipettes were then added to the challenged organisms. The single challenger drop contained up to 10^4 organisms. Duplicates of the challenger and challenged lines at time of challenge were homogenized and viable counts performed for each. These were then killed with formaldehyde. The test mixtures, plus controls for challenger and challenged lines alone,

were incubated under various environmental conditions at 37°. Broths, agars and shaken cultures were tested at 6, 24 and 48 hr. on individual samples for each hour.

At count times, test broths were homogenised and serial tenfold dilutions were made in 4.5 ml. amounts of saline. The growth from plates was harvested in 20 ml. saline per plate, homogenized and diluted. 0.1 ml. of each dilution was quickly pipetted and spread on (a) rhamnose (EMB) eosin methylene blue agar and (b) rhamnose (DM) minimal agar supplemented with broth. These were incubated at 37° for 2 and 3 days respectively before counting.

Tests and controls of challenger and challenged lines were killed with formaldehyde. Measurements of turbidity, pH, total count, haemagglutinating activity (HA) and power (HP) were recorded. Chosen preparations were examined by electron microscopy. The final 48 hr. population was always screened. This involved, in certain cases, testing 24 each of pale and pink colonies from EMB for HA. and fimbriae. Other strains involved more extensive characterisation.

EXPERIMENTAL AND RESULTS

SECTION I

Genetic control of fimbriation: evidence for complex loci.

Of the Salmonella typhimurium strains collected by Professor J.P. Duguid, 132 strains belonged to the group classified in the introduction as FIRN, and one strain Sa 6925 was called FIRN' (i.e. fim-, inl+ and rha-), and included with FIRN for all further investigations. These naturally-isolated non-fimbriate strains were, as previously mentioned, representative strains spanning the years from 1948 - 1963, and of diverse phage-types and fermentation groups. All FIRN strains were characteristic in their sugar spectrum, fermenting in peptone water the following sugars: trehalose in 10 - 24 hr., xylose in 10 - 24 hr., rhamnose in 3 - 40 days and not fermenting inositol. Those strains, with very rare exceptions, did not produce inositol-fermenting mutants, and never spontaneously reverted to a fimbriate state even on continued serial subculture in nutrient broth. The 80 per cent. majority of Salmonella typhimurium strains called FIRP were also characteristic in fermentation: trehalose in 10 hr. - 7 days, xylose in 6 hr. - 5 days, rhamnose in 6 - 8 hr. and in many cases inositol in 10 - 24 hr. Serial broth subcultures selected the fimbriate form, the presence of fimbriae being detected by the tile haemagglutination test, mannose-sensitivity test, and by the electron microscope (J.P. Duguid, personal communication).

It was planned to investigate the genetic control of fimbriation and the high correlation of apparently unrelated characters observed in these non-fimbriate mutants by means of

transduction techniques. Preliminary work by Duguid, Stocker and Hume (unpublished results), had been done with a small number of these strains and it was, therefore, known that the gene controlling fimbriation could be transduced. The analysis of this research is now laid out, and some interpretations and conclusions made on the available data.

Transduction of non-fimbriate FIRN strains of Salmonella typhimurium to fimbriation.

Phage P22 (Zinder and Lederberg, 1952) was used as seed phage to propagate lysates on a range of donor Salmonella typhimurium FIRP and FIRN strains, satisfactory lysates being of the order of 10^{10} p.f.p. per ml. The recipient non-fimbriate FIRN strains were incubated with lysates at multiplicities from 1 - 5.

Choice of a selective medium.

Although the presence of fimbriae at the cell surface endows an organism with agglutinating properties, it is not understood if their presence bestows other advantages. Since it is unlikely that an organism as small as a bacterium will burden itself with the synthesis of non-essential organs, we must accept provisionally in the absence of conflicting evidence, that the property of adhesion to substrates represents the main function of fimbriae. This, however, poses the problem of devising a selective medium for the detection of fimbriate transductants, because, except for atypical colonial differences in certainstrains, there is no direct method of differentiating the two types of cells. However, fimbriate organisms in

static broths under aerobic conditions, characteristically outgrow non-fimbriate organisms by their pellicle-forming ability, (Duguid and Gillies, 1957, Duguid and Wilkinson, 1961), which suggested serial broth subculture as an efficient, if not absolute, selective medium. Although the number of lysogenic, non-fimbriate recipients surviving the lethal consequences of phage addition greatly exceeds the number of potentially fimbriate transductants, such manipulation has proved effective and selective for the isolation of fimbriate cells from a transduction mixture.

Behaviour of non-fimbriate strains treated with phage lysates and incubated in static, aerobic broth.

With the exception of the non-motile strains Sa 1292, 1645, 1714 and 1720, all FIRN strains, including the FIRN[1] strain, were susceptible to transduction to fimbriation with lysates from a number of different donors. Not only were tests made with lysates from wild-type fimbriate donors but parallel tests were made with lysates from other FIRN strains, since recombination of two inactive genes to give a fim+ transductant would present evidence of non-identical alleles and differing genotype among different FIRN strains. "No-phage" controls were included to observe the behaviour of test strains of FIRN class in the absence of transducing phage, and confirm that they never underwent spontaneous mutation to the fim+ state. Phage and broth controls were always run in parallel with the tests, and, in their absence or contamination, positive results could not be considered significant. The ease of transduction varied

among the different FIRN strains. A number proved susceptible on first testing, and usually were not retested, while others were transduced with difficulty and only on repeated testing.

Factors controlling the success of transduction to fimbriation.

(I) Selective methods for the isolation of transductants are usually such that only cells altered by the incorporation of the desired character are favoured, e.g. transduction to prototrophy and flagellation. In these cases, large numbers of recipient cells can be transduced, and the non-transduced members when plated on the selective medium do not interfere since only the transduced cells grow. However, in the initial hours in broth, both fimbriate and non-fimbriate grow equally well, and so to favour the selection of fimbriate transductants, the recipient cells were grown only to a cell concentration of 2×10^8 per ml. before the addition of phage.

(II) Duguid (personal communication) has shown that the percentage number of fimbriate cells in a population increases greatly with successive broth subcultures, and so to ensure the detection and make easier the isolation of fimbriate clones, tests were continued for two, and, occasionally, three 48 hr. subcultures in broth.

(III)Certain FIRN strains, transducible only on repeated exhaustive testing, were known to be lysogenic for P22 or other related type A phages - Boyd and Bidwell (1957). This results in a reduction in the transduction frequency (Lennox, 1955).

(IV) Certain strains, e.g. Sa 932, proved difficult to transduce to any marker tested - fim, rha, inl. - and seemed unable to adsorb phage as completely as other strains. These strains may have been unsuitable in other ways.

(V) Certain fimbriate strains were not efficient donors of fimbriation, e.g. Sa 206 and Sa 1404, although tests showed their efficacy in transducing other genes such as rha fla and thr. The reason for this is not known.

(VI) Certain non-motile strains were not transduced to fimbriation even on repeated testing, and the reason for this will be discussed fully later, with reference to motility in the recipient strain.

The accompanying table 3 summarises the results of all tests in which FIRN strains were treated with transducing lysates from fimbriate and FIRN donors, and shows that transduction is readily detected. The overall transduction efficiency, or percentage number of successful tests, is 28% for FIRN recipients treated with fimbriate donors. This low figure of 28% is partially explained by the substantial number of tests made with poor lysates and poor recipients. If one considers the transduction efficiency for only good donors and good recipients, a much higher rate of 58% is found, (These two terms are, of course, quite arbitrary). From every positive test a fimbriate transductant strain was isolated in pure culture, as indicated in the methods section, to allow the analysis of its sugar spectrum with the four key sugars used to differentiate FIRP and FIRN, viz. trehalose, xylose, rhamnose

Table 3. Transduction of fimbriation to motile *Salmonella typhimurium* FIRN strains: aggregated results.

No. and class of recipient strain	No. of tests giving transduction to *fim* + / no. of tests performed with phage prepared on donor strain that was:		No. of tests giving mutation to *fim* + / no. of tests made without added phage. i.e. "no-phage" controls.
	Fimbriate	FIRN *	
126 FIRN strains and 1 FIRN/ strain	383/1197	0/848	0/720

FIRN/ = Inositol fermenting strain Sa6925, otherwise typical FIRN type.

* Different FIRN strain from the recipient strain.

In these experiments, 20 fimbriate and 34 FIRN donors were tested.

For detailed results, see Appendix, table A.

and inositol. All screenings of this nature proved that the transductants were fimbriate derivatives of the original FIRN strains, and not casual contaminants, the fim+ strain having the two typical FIRN characters, rha- and inl-. Electron microscopy of a number of strains (in all 36 fimbriate transductants) revealed that the haemagglutinating transductants were typically fimbriated cells.

The high degree of all-or-none correlation between the characters fim and rha had suggested that they might be genetically controlled at a single locus or closely related loci. Therewas, however, no evidence in any of the fimbriate transductants, selected and screened quite randomly, for donation of the two markers in a single-step transduction, showing that these two loci are not linked on the circular chromosome of Salmonella typhimurium.

Stability of the transductants.

Six strains of fimbriate transductants which had been isolated some months previously and stored on Dorset egg slopes were examined for the stability of the fim character transduced. The strains tested were Sa 654, 703, 809, 1288, 1642 and 6785. Each was plated on nutrient agar and four colonies of each strain selected - two for serial subculture on agar, two for subculture in broth. After 10 serial broth subcultures, each was plated to give single colonies on nutrient agar and six colonies from each picked into broth and incubated for 48 hr. for separate testing. All 72 individual colonies tested were fimbriate. After 20 agar subcultures, six colonies from

each plate were subcultured into broth for individual testing. Again, all were fimbriate.

Calculation of the percentage fimbriate population at first and second forty-eight hour broth subcultures.

The haemagglutinating activities of the first and second 48 hr. subculture deposits were estimated arbitrarily as +, ++, +++ and ++++ dependent on the speed at which agglutination was observed in tile tests. To evaluate the percentage number of fimbriate cells at any given time, a positive haemagglutinating deposit was resuspended in broth after testing, and plated in duplicate on nutrient agars to give single colonies, which were picked 50-100, with a straight wire into nutrient broths. Their deposits, after 48 hr. incubation, were tested for haemagglutination. Seven such experiments with five different recipients showed that the population of fimbriate transductant cells after the first 48 hr. subculture varied from 5 - 8% for a HA. result of +, 14 - 24% for a HA.result of ++, to 20 - 33% for a +++ HA. reaction. By the end of the second 48 hr. broth, the final population of fimbriate cells varied from 90 - 95%.

Calculation of the transduction rate to fimbriation.

In the absence of a medium absolutely selective for fimbriate cells, it is impossible to evaluate accurately the transduction rate on a quantitative basis. An approximate was gained by the following procedure. Twelve 9 ml. nutrient broths were seeded with a good recipient

strain, Sa 577, and 1 ml. amounts of donor phage, prepared on S.typhimurium Sa 744, were added when the recipient cultures first became visibly turbid. FIRN strains at such a time gave viable counts ranging from 95 - 130 x 10^6 cells per ml. The donor lysate contained 1.0 x 10^{10} p.f.p. per ml., and so the multiplicity of infection would be of the order of 10. After 15 min. incubation to allow complete phage adsorption, serial ten-fold dilutions of these original phage-bacterium mixtures were made to 1:100 in 9 ml. amounts of broth. The original twelve tests and their corresponding dilutions were afterwards incubated aerobically and statically for 48 hr., second broth sub-cultures made, and the deposits of all tubes tested for haemagglutinating activity. Such dilution was hoped to indicate the number of potential fimbriate transductants in the original mixture, by the number of diluted tests which became positive. Of the original twelve tubes, eight from twelve were strongly positive, but no diluted tubes revealed haemagglutination. This experiment was repeated and confirmed for another strain, suggesting that the total number of fimbriate cells is not greater than ten, and, therefore, that the transduction rate is of the order of 5 x 10^{-7} to 1 x 10^{-8}.

The importance of motility in the recipient strain.

The only strains that have not been transduced to fimbriation were Sa 1292, 1645, 1714 and 1720. In addition, certain of the Sa 100 series were not transduced: these were SL 43, SW 573, SW 578 and SW 580. The results for all

Table.4 Transduction of fimbriation to non-motile *Salmonella typhimurium* FIRN strains: aggregated results.

Reference no. of non-motile recipient strain	No. of tests giving transduction to *fim* + / no. of tests performed with phage prepared on donor strain that was:		No. of tests giving mutation to *fim* + / no. of tests made without added phage. i.e. "no-phage" controls.
	Fimbriate	FIRN	
Sa 100 *	0/22	0/12	0/11
Sa 635	1/87	0/64	0/33
Sa 1292	0/9	0/13	0/9
Sa 1645	0/11	0/11	0/3
Sa 1714	0/26	0/4	0/3
Sa 1720	0/27	0/4	0/2
Total	1/182	0/108	0/61

* Sa 100 = non-motile strains SW573, 578, 580 and SL43.

In these experiments, 12 fimbriate and 15 non-fimbriate donors were tested.

For detailed results, see Appendix, table A.

the non-motile strains in the collection, including the non-motile FIRN Sa 635, which was transduced on a single occasion, are interesting because they supply a partial explanation for the low transduction efficiency of 28% for FIRN strains as recipients. These results are shown in table 4.

Meynell (1961) had shown that the phage ØX/was capable of attacking only those Salmonella strains with functional flagella, not of the antigenic series g-complex. No such explanation as non-motile FIRN strain inability to adsorb phage P22 can be advanced here since Stocker et al (1953) had successfully employed this same phage to transduce flagellation into many Salmonella O types of group B of the Kauffman-White scheme. This was verified for these non-motile FIRN strains by means of phage-adsorption studies as outlined in the methods section. The strains tested in this fashion were SL 43, SW 578, SW 580 and SA 635, all of which adsorbed phage P22 when infected at a multiplicity of I.

The importance of these findings justified further investigations and, to this end, a number of strains was prepared for transduction to fimbriation. The strains used were non-flagellate and non-motile, flagellate and non-motile (i.e. paralysed flagella), and flagellate and motile. A motile derivative of Sa 635, numbered Sa 1183, was received from Dr. B.A.D. Stocker (SL 667/G 434). This strain was a transductant isolated from Sa 635 using phage grown on S.typhimurium LT-2; it was non-lysogenic and still sensitive to P22. Spontaneous motile mutants of strains

Table 5. Transduction of fimbriation to non-motile and motile pairs of *Salmonella typhimurium* FIRN strains: aggregated results.

Recipient strain reference no.	Flagella	Motility	No. of tests giving transduction to *fim* +/no. of tests performed with phage prepared on donor strain that was:		No. of tests giving mutation to *fim* +/ no. of tests made without added phage.
			Fimbriate	FIRN	
SW 573	−	−	0/6	0/1	0/2
SW 578	+	−	0/6	0/7	0/5
SW 582	+	+	11/12	0/4	0/5
SW 580	+	−	0/6	0/1	0/2
SW 583	+	+	4/7	0/4	0/4
SL 43	+	−	0/4	0/3	0/2
SL 43S	+	+	3/14	0/10	0/9
Sa 635	−	−	1/87	0/64	0/33
Sa 1183	+	+	6/14	0/4	0/4

SW 578 is original "paralysed" flagellate isolate of Friewer and Leifson (1952) and SW 573, 580, 582, SL 43 and SL 43S are variant strains derived from it.
SW 580 is N.C.T.C. 8298.
SL 43 is a flagellate mutant of SW 573, Stocker *et al* (1953).
SW 582, 583 and SL 43S are spontaneous motile derivatives of SW 578, 580 and SL 43 Meynell, (1961).
Sa 635 is a wild-type FIRN strain (Duguid, unpublished results) and Sa 1183 a non-lysogenic, motile transductant of Sa 635 obtained by Dr. B.A.D. Stocker.

SL 43, SW 578 and SW 580 were supplied by Dr. Elinor Meynell. The motile derivatives are included in the Sa 100 series and numbered, respectively, SL 43S, SW 582 and SW 583.

The results of the attempted transduction to fimbriation with these pairs of non-motile and motile derivatives of a single strain are interesting when viewed in parallel - table 5. The results indicate that recipient cells must be both flagellate and motile before successful transduction is detected at a significant rate. These results confirm the findings of Hume (1961, personal communication). Failure to transduce other non-motile strains to fimbriation has been encountered, e.g. Salmonella gallinarum (Old, unpublished results).

Preliminary transduction to flagellation for use as recipients was not generally resorted to, because, since this results in lysogenisation in the P22 system, the transduction efficiency is significantly decreased, Lysogenic motile cells from non-motile FIRN parents have yielded only one fim+ transductant. One obvious point arising from consideration of the date in table3is that each FIRN strain was not tested by reciprocal transduction with lysates from every other FIRN. The somewhat tedious process of selection and characterisation of successful fimbriate transductants, and the low transduction rate, make this an impossible task. In addition, satisfactory lysates could not be prepared on many strains, perhaps because of their lysogenic nature. Induction might have been successful but neither materials nor time were available in sufficient

quantity. Nevertheless, some forty FIRN strains have yielded satisfactory lysates and these, although tested against only a few other FIRN strains, do give a satisfactory, if only indicative, picture. In no case was a fim+ transductant obtained by recombination between two FIRN strains. In nearly eight hundred "no-phage" controls, no FIRN strains mutated to wild-type, suggesting that the defect in FIRN genotype is not due to a single, point mutation, unless that site is stable.

Are non-fimbriate crosses ever fertile?

At the outset of this investigation, only four strains were available which did not conform to the rule which correlated fimbriation and rhamnose characters. These strains were Sa 519, 749 and 750 each of which is non-fimbriate, inositol and rhamnose fermenting (fim- inl+ rha+). There was too strain Sa 619 which is non-fimbriate, inositol non-fermenting and rhamnose fermenting (fim- inl- rha+). This could not be positively identified as a Salmonella typhimurium since it was a monophasic flagellate strain, lacking antigen i. Transduction analyses of the type carried out by Lederberg and Edwards (1953) might have revealed its overt antigens. Widespread search for other such unusual strains produced only two more examples, viz. Sa 1436, which was non-fimbriate, inositol and rhamnose-fermenting and Sa 6631, non-fimbriate, inositol non-fermenting and rhamnose-fermenting. Strain Sa 1566a, received from Dr. P.R. Edwards, spontaneously threw off non-fimbriate mutants (Sa 1566b). These like the parent were inositol

Table. 6. Characters, source and date of rare non-fimbriate strains of *Salmonella typhimurium* that are not FIRN.

Sa Reference No.	Supplier and Supplier's no.		Source	Date	Place	Colindale phage types.		Fermentation, within 24 hr., of	
						Old	New	Rhamnose	Inositol
Group A.									
519	N.C.T.C.	8392	Man	1934		U	U	+	+
749	L.O.Kallings.	Lt-13		1960	Sweden	5	ul19	+	+
750	"	LT-13L		"	"	5	ul19	+	+
1436	B.A.D.Stocker	2M 140	Mutton	1962	N.Zealand	u	u38	+	+
Group B.									
619	J.E.Wilson.		Fowl	1955	Scotland	u	u	+	-
1566b	P.R.Edwards.		Pig	1962	U.S.A.	1a	u57	+	-
6631	E.R.L.		man	1958	England	2	10	+	-

non-fermenting and rhamnose fermenting. From the original preservation slope, of 59 individual clones examined, 50 were fimbriate Sa 1566a and 9 were Sa 1566b. The nine latter were subcultured serially through twenty nutrient broths and were quite stable, never reverting to the fimbriate state. Each sub-type was phage-typed as 1a/u57.

Classified according to their inositol-fermenting character, the seven strains are divided into two groups, A and B, shown in the table 6. Allelic recombination by two recessive mutants to wild-type full activity is a characteristic phenomenon of all gene loci studied to date (Demerec and Hartman, 1959). It has been more readily observed when the groups show biochemical diversity (Hartman, Loper and Serman, 1960). Among these non-fimbriate mutants, therefore, we might find some evidence of complex loci in the segment controlling expression of fimbriation, which had eluded us among the apparently identically-mutated FIRN strains.

Transduction studies with strains of groups A and B.

Group A.

Sa 519 This strain has not been transduced to fimbriation with donor lysates prepared on wild-type fimbriate or FIRN strains, nor with lysates from any other strain in Group A or B. Although Sa 519 is non-motile, spontaneously isolated motile mutants were used as recipients also without success. This strain was mentioned in the introduction because of its ability to mutate spontaneously to a form colonially different from Sa 519, which is large, smooth and non-

fimbriate. The mutant Sa 519HA on agar produces small, compact colonies which are haemagglutinating, due to the presence of fimbriae (Duguid, personal communication). The parent Sa 519 was tested with the known active lysate from a good donor Sa 497 under microaerophilic conditions, with reduced medium depth and a temperature of 30°, all of which are favourable factors in aiding the outgrowth of fimbriate cells. None of these resulted in the isolation of fim+ transductants. The reason for such failure must lie elsewhere than in motility. Lysates prepared on donor Sa 519HA have evoked fimbriate transductants from Sa 519. These like the donor are of small colony form. This calls to mind the small colony forms of suppressor mutants (Loveless and Howarth, 1959).

Sa 749 and Sa 750. These two strains were found to be resistant to all but one among the twelve type A phages of Salmonella typhimurium. After passage through these strains, phage A2c was suitable for propagation of lysates of sufficiently high titre. One strain is factor I positive, i.e. it already carries or is lysogenic for an A-type phage (Stocker, personal communication). It was found that these two strains could be transduced to fimbriation with phage lysates prepared on fimbriate donors. The transduction efficiency was low - 10% for Sa 749 and only 2% for Sa 750. Nevertheless, despite this decrease in transduction efficiency and the fact that two defective genotypes will recombine at a significantly less detectable rate than

when only one strain is defective, we have been able to isolate fimbriate transductants from Sa 749 and Sa 750 when these strains were treated with transducing lysates from FIRN strains as donors (Sa 577, 704, 816 and 6799). In all cases, subcultures were continued until fimbriate transductants were detected in numbers large enough to allow their isolation; sometimes three 48 hr. subcultures were required. In later experiments with the strains of groups A and B, they were grown for seven-day periods to allow maximum opportunity for the expression and outgrowth of fimbriate cells. Transductants of Sa 749 and Sa 750 could be reliably checked by their sugar spectra, because, although transduced to what is virtually FIRP status, they gave late, mutational fermentation of xylose (e.g. after 7 - 21 days). This character was found in all Sa 749/750 fim+ transductants. No fimbriate transductants were obtained in cross transduction experiments between Sa 749 and Sa 750, even on repeated testing. Strains Sa 519 and Sa 1436 also did not give fimbriate transductants in numerous reciprocal tests with these two strains. Both strains gave occasional fim+ transductants when treated with lysate from Sa 619 of group B, and, in addition, Sa 750 did so with lysates from Sa 6631.

Sa 1436. This strain, like the previous two, was transduced to fimbriation by both fimbriate and non-fimbriate donors. The transduction efficiency between Sa 1436 and fimbriate donors was 12%. FIRN lysates (Sa 611, 635 and 816) gave

fim+ transductants with Sa 1436, after two one-week subcultures. Strains of group B, were, as far as tested, not capable of recombination with Sa 1436. Group A strains thus present a wealth of information. Sa 519, because of its poor motility and transducibility only by a mutant from itself, is best considered separately. Strains Sa 749, Sa 750 and Sa 1436 obviously represent a transduction class distinct from the other non-fimbriate groups, B and FIRN. The frequent detection of mutants in reciprocal crosses agrees with this statement. These three strains, although functioning well as recipients in reciprocal crosses with FIRN strains, are not capable of acting as efficient donors of fimbriation to recipient FIRN strains. This mutation involved in group A is obviously different from that of the FIRN series.

Group B.

Sa 619. This monophasic strain has been transduced on only one occasion, even on repeated testing with a known efficient donor lysate from S.typhimurium Sa 497. Reciprocal tests with all other lysates revealed no fimbriate transductants. Lysates on Sa 619, however, did on two occasions transduce fimbriation into strain Sa 749 and Sa 750, but not to any member of group B.

Sa 6631. This strain, like Sa 619, was transduced to fimbriation only by lysates from fimbriate donors, those from non-fimbriate strains being ineffective. As a donor it gave recombination to fimbriate wild-type with Sa 750, and on a singl e occasion with Sa 1566b, but not to any other

Table 7. Transduction of fimbriation to *Salmonella typhimurium* strains of groups A and B: aggregated results.

Recipient strain: Sa reference no.	No. of tests giving transduction to *fim* +/ no. of tests performed with phage prepared on donor strain that was:								No. of tests giving mutation to *fim* + / no. of tests made without added phage. i.e. "no-phage" controls.
	Fimbriate *	Group A				Group B			FIRN *	
		519	749	750	1436	619	1566b	6631		
519	0/80	0/7	0/6	.	0/6	0/2	0/6	0/7	0/72	0/77
749	15/142	0/7	.	0/18	0/19	1/2	0/11	0/13	9/153	0/83
750	3/138	0/7	0/24	.	0/19	1/2	0/11	1/13	4/149	0/79
1436	9/77	.	0/7	0/20	.	.	0/6	0/18	3/38	0/13
619	1/20	0/2	.	.	.	.	0/6	0/6	0/4	0/3
1566b	16/33	.	.	4/6	5/7	0/2	.	1/6	27/34	0/10
6631	2/3	.	0/6	.	0/6	0/8	0/8	.	0/15	0/3

* 18 fimbriate and 29 FIRN donors were tested.
Sa 519 is poorly motile.

members of groups A or B or to the FIRN group.

Sa 1566b. This was the most valuable isolate of the seven, and its suitability both as donor and recipient became clear. Sa 1566b has been transduced to fimbriation by lysates from fimbriate donors and also, readily, by non-fimbriate FIRN and group A strains. Limited tests with other group B members did not give fim+ transductants. The results of the experiments with these seven strains as recipients are collected in table 7, in which results with lysates from fimbriate and FIRN donors are expressed collectively. That groups A and B represent two transduction classes as well as biochemical classes is clear from the table.

Artificially isolated non-fimbriate mutants of Salmonella typhimurium.

A series of rhamnose non-fermenting mutants derived from S. typhimurium LT-2 line by ethyl methane sulphonate treatment was obtained from Mrs. Jean Dubnau at The Lister Institute of Preventive Medicine, London. Two of these strains proved stable enough in the fim- character for transduction work; their Sa numbers were 1134 and 1137 (i.e. Dubnau strains 464 and 461 respectively). The original parent from which the progeny was derived was SL 680 (LT2 ade-pro-H_I^{iMI0} fla-str-r-ile-fim-). In addition, since non-motile strains function only as donors in transduction to fimbriation, a number of motile derivatives was obtained (by recombination using colicinogenic agents) for use as recipients in reciprocal transduction testing.

Table. 8. Transduction of fimbriation to Salmonella typhimurium strains of FIRN Groups A and B with transducing lysates from non-fimbriate mutant Dubnau strains.

Recipient strain, class and Sa ref.no.	No. of tests giving transduction to fim+ / no. of tests performed with phage prepared on donor strain that was:		
	18	1134	1137
132 FIRN strains and 1 FIRN' strain	6/10	43/86	134/184
519	.	0/3	.
749	0/2	2/22	.
750	0/2	1/22	.
1436	.	4/7	.
619	.	.	.
1566b	.	0/23	.
6631	.	2/10	.

Dubnau strains Sa 1134 and 1137 were obtained from Mrs. Jean Dubnau from Salmonella typhimurium LT-2 by ethyl methane sulphonate treatment - see page 109.

Strain 18 is a motile recombinant obtained by Miss Sylvia Smith see page 110.

Miss Sylvia Smith, The Lister Institute of Preventive Medicine, London, selected five recombinants for fla+ from the donor strain (LT-2 met-try-$H_1^b H_2^{enx}$ azi-gal.) and for streptomycin resistance from the Sal134-1137 line. Of the five recombinants, only Dubnau 18 proved stable enough in its fim- character for use in transduction. The other strains reverted spontaneously to fimbriation on serial broth subculture, so that evaluation of true transduction was impossible since the complete experimental procedure lasts for four days.

Lysates prepared on Dubnau strains 18, 1134 and 1137 transduced fimbriation into the non-fimbriate FIRN group at a very high rate. The Dubnau strains, being non-lysogenic for P22 gave readily attainable high-titre lysates. Group A and B strains behaved similarly to the FIRN strains and fimbriate transductants were obtained from strains Sa 749, 750, 1436 and 6631. (see table 8).

As a recipient, strain Dubnau 18 was also efficient, being transduced by fimbriate and non-fimbriate donor lysates at a frequently detectable rate. An interesting feature soon emerged from repeated cross testing between Dubnau stfains and Sa 1566b, with each tested as donor and recipient, because in all of thirty-three significant tests negative results were always recorded and no fimbriate wild-type detected. This consistent record of negative results is unusual for two strains whifh individually are efficient as both donor and recipient. It would seem that the

naturally isolated strain Sa 1566b and the EMS-treated Dubnau mutant have a common mutation with respect to fim. Thus, although in table 6 Sa 1566b was placed with Sa 619 and 6631, its obvious inability to recombine with Dubnau strains conflicts with the properties of the other two strains, and suggests that it should be placed with the Dubnau strains in a separate class.

In an attempt to solve the problem of interrelationships among the FIRN strains the problem was next viewed from their behaviour when treated with transducing lysates and selected for rha.+.

Transduction of FIRN strains of Salmonella typhimurium to rhamnose-utilising ability.

In this instance, where a good selective medium is available in the form of rhamnose minimal medium and a high transduction rate is known for this gene (Englesberg, and Baron, 1959), the problem was an easier one. Transducing Lysates were prepared on rhamnose-fermenting donors (strains of FIRP, group A and B) with which it was possible to transduce all 132 FIRN and 1 FIRN[1] strains to rha+. The non-motile strains were not resistant to this transduction, although certain strains, e.g. Sa 932, again proved as difficult to work with as when being transduced to fimbriation. Only lysates from rha+ strains were effective, and, again, comparable to, and confirmatory of, the fimbriation results, only one character per test was transferred, i.e. rha+fim+ cells never arose from FIRN strain by a single-step co-

Table 9. Transduction of rhamnose-utilising ability to *Salmonella typhimurium* FIRN strains: aggregated results.

No. and class of recipient strain.	No. of tests giving transduction to *rha* +/ no. of tests performed with phage prepared on donor strain that was:			No. of tests giving mutation to *rha* +/ no. of tests made without added phage. i.e. "no-phage controls.
	Rha +	Dubnau – *Rha* –	FIRN – *Rha* –	
132 FIRN strains and 1 FIRN' strain.	178/309	61/338	0/250	0/269

FIRN' = Inositol fermenting strain Sa6925, otherwise typical FIRN type.

In these experiments, 9 rhamnose-utilising strains and 22 FIRN strains were used as donors.

For detailed results, see Appendix Table B.

transduction. Since it is difficult to test a large number of single cells for fimbriation, up to twenty single rha+ colonies were inoculated into a single nutrient broth. In this way, large numbers of cells could be screened using a minimum number of broths. In no instance did any broth inoculated with such numbers of colonies become positive.

The results in the accompanying table 9 show that there was no recombination among rhamnose deficient genes of FIRN origin. The FIRN reciprocal crosses are incomplete but suggestive of no cross-reaction. If fertile inter-FIRN crosses were not detected in fimbriation transduction because of limitations in the selective mechanism, then, had such differences in the FIRN strains been present, small numbers of rha+ transductants should have been readily detected on rhamnose minimal medium supplemented with broth. No such transductants arose from inter-FIRN crosses. Tests with the Dubnau strains mutated at a different site from FIRN, yielded rhamnose transductants in small numbers when crossed with FIRN strains. Evidence of pseudo-allelism among other rhamnose non-fermenting strains - Sa 1179, 1180, 1541, 6768 and 6899 - was shown by reciprocal transductions among these five strains. The number of rha+ recombinants obtained was usually of the order of one or two per test. The results are presented in table 10.

Mutagenic response of non-fimbriate strains of Salmonella typhimurium when treated with three potent mutagens.

Z. Hartman (1956) showed that approximately 60% of Salmonella typhimurium mutants were mutagen stable,

Table 10. Reciprocal transductions to *rha* + among five fimbriate, rhamnose non-utilising strains of *Salmonella typhimurium*.

Recipient strain Reference No.	Transduction to *rha* + with lysate prepared on donor strain that was rhamnose non-utilising:-				
	1179	1180	1541	6768	6899
1179	-	-	+	+	+
1180	-	-	+	+	+
1541	+	+	-	+	+
6768	+	+	+	-	+
6899	+	-	+	-	-

+ = Small numbers of rhamnose utilising recombinants detected.

- = No recombinants detected.

i.e. the rate of back mutation to wild-type was not increased by mutagen treatment. She also found that stability of a locus was correlated with the lineage of a strain, because all mutagen stable types were LT-2 derivatives. The various genes, including non-identical alleles of the same locus react specifically to each mutagen so that specificity and stability are both variable factors. Similar findings were proposed for Escherichia coli mutants which differed in being more mutagen labile (Glover, 1956). Demerec and his co-workers (1958) confirmed these findings, and showed that the stable and labile sites within any locus are randomly arranged. They also discovered a mutability factor - a gene - responsible for the typically high mutability of S. typhimurium and found evidence for its existence in relation to ten gene loci.

All the non-fimbriate strains in this collection were known not to mutate spontaneously to the wild-type state, and so any mutant from which revertants were induced by treatment with a mutagen would be mutagen labile. In the hope of proving constant stability or lability for all the FIRN strains and so prove their identical nature, three mutagens were chosen, viz. ultraviolet light, manganous chloride and ethyl methane sulphonate. (In a smaller number of experiments ethyl methane sulphonate was used, since it is claimed to produce high yields of revertants and slight lethal effects (Loveless and Howarth, 1959).

All FIRN strains were tested with ultraviolet light

in single tests, with an average death rate of 90%. Each strain was also tested from four to six times with manganous chloride. The following strains reverted to fimbriation after treatment with manganous chloride: Sa 629, 632, 874, 932 and 1577. Ultraviolet light produced a revertant from Sa 1376, while ethyl methane sulphonate yielded no mutants. The sugar spectrum of several clones of each revertant was checked and shown to have the characteristic FIRN markers, rha- and inL-. Slide agglutination with specific antisera confirmed them as Salmonella typhimurium strains. The demonstration of MS fimbriae showed that this treatment had not resulted in the production of MR haemagglutinins. The revertants from FIRN strains, Sa 629, 874 and 1577, were shown to possess normal fimbriae when examined by electron microscopy.

The other non-fimbriate groups, when tested in like fashion, yielded 40% of mutagen labile strains. Sa 749 reverted on treatment with both mutagens and the fimbriate clones isolated, screened and shown to be xylose late-fermenting strains. Whereas FIRN revertants were detected usually at the second or third subculture, Sa 749 was always detected at the first subculture. Sa 1436 reverted after manganous chloride treatment, and so too did Sa 1566b. Two other strains should be mentioned here; Sa 848 and Sa 1451, which were rhamnose-fermenting and occasionally gave traces of mannose-sensitive haemagglutination. This did not increase on serial broth subculture. Even although fimbriae

were scanty and detected only in a few cells, these strains were regarded as FIRP. Each strain on treatment with mutagens revealed its ability to produce strongly-fimbriate revertants, detectable after 48 hr. subculture. These two strains may have been mutagen labile, single-site mutants but attempts to use them as recipients in transduction to fimbriation gave irregular results.

Linkage of fim gene to other chromosomal markers.

Rhamnose - the many fim+ and rha+ transductants tested had never been simultaneously transduced to the other marker.

It was interesting to find whether the gene controlling fimbriation was linked to any other marker as detected by transduction. This was done only in relation to those markers which had suggested possible linkage from the current investigations.

Inositol - the possibility that inl might be linked genetically suggested itself from consideration of the preliminary characters of Salmonella typhimurium strains, because inositol non-fermentation was correlated to non-fimbriation (J.P.Duguid, personal communication). In addition, certain fimbriate strains were inositol negative, e.g. Sa 206 and SaI404, and these proved to be poor donors of fimbriation, although they transduced other markers, such as rha, efficiently.

Occasionally, fim+ transductant FIRN strains were also inositol-fermenting. This was found, in all, on about ten occasions, but too irregularly for any quantitative assessment

Table 11. Transduction of inositol-utilising ability to selected Salmonella typhimurium FIRN strains.

Recipient Strain - Sa ref. no.	No. of tests giving transduction to inl+/ no. of tests performed with phage prepared on donor strain that was:											No. of tests giving mutation to inl+/no. of tests prepared without added phage.
	inositol-fermenting							inositol-non-fermenting				
	FIRP and Group A					Dubnau		FIRP	FIRN			
	375	497	742	749 *	1302 *	1134	1137	206 ♦	100 x	614	622	
645	0/2	1/1	.	1/1	1/1	0/2	0/1	.	0/1	0/1	0/1	0/6
652	0/2	0/1	.	1/1	1/1	0/2	0/1	0/1	0/1	0/1	.	0/6
705	1/2	.	.	.	.	.	.	0/1	.	0/1	.	0/2
706	0/1	1/2	.	1/1	1/1	1/2	0/1	0/1	.	0/2	.	0/6
713	1/1	.	.	.	.	.	.	.	0/1	,	.	0/1
715	0/2	0/1	.	1/1	0/1	0/2	0/1	0/1	.	0/1	.	0/6
735	1/1	0/1	0/1	.	.	.	.	0/1	.	.	.	0/2
738	0/1	1/2	0/1	1/1	1/1	0/2	0/1	.	.	0/1	0/1	0/6
760	1/2	.	.	.	.	.	.	0/1	0/1	.	.	0/2
805	0/1	0/1	.	.	.	.9	0/1	0/1	.	0/1	0/1	0/3
850	0/1	0/1	.	1/1	0/1	0/1	0/1	0/1	.	0/1	0/1	0/5
6683	0/1	0/2	0/1	1/1	1/1	0/2	0/1	0/1	.	0/1	.	0/6
7471	0/1	0/2	0/1	1/1	1/1	0/1	0/1	.	.	0/1	0/1	0/6

* Transductants isolated in inositol peptone water, all others on inositol minimal agar.

x Sa 100 = S143S.

♦ Sa 206 is an inositol non-fermenting FIRP strain.

to be made. The origin of spontaneous inositol-fermenting mutants in a fashion analagous to the rhamnose-fermenting mutants (Duguid, unpublished results) and xylose-fermenting mutants (Stocker, personal communication) was detected only rarely in many experiments devised to isolate them. To investigate the phenomenon more definitively, thirteen FIRN strains, chosento represent a range of phage-types and fermentation groups, were used as recipients. The following strains Sa 645, 652, 705, 706, 713, 715, 935, 738,760, 805, 6683 and 7471 were transduced to an inositol-fermenting state with difficulty, because of the unsuitability of inositol as sole carbon and energy source in a minimal medium. Difficulty with solid media led to the successful use of inositol peptone water for selection purposes, see table 11.

All inositol-fermenting transductants from FIRN strains were checked by their ability to ferment inositol in peptone water within twelve hours, and on inositol EMB agar in 24-48 hr., and by their utilisation of inositol in minimal medium. Their FIRN origin was proved by being non-fimbriate and rhamnose non-fermenting. The isolation of transductants which were fimbriate and inositol-fermenting (i.e. evidence of fim-inl cotransduction) never occurred.

Experiment devised to detect inositol (or rhamnose) fimbriate FIRN transductants.

The task of screening large numbers of any fimbriate transductants is difficult because it is consuming of time and materials. If such a cotransduction of fimbriation

with either of these two characters occurred at a low rate, then large numbers of cells from 10^2 - 10^4 would have to be individually tested to detect occasional cotransductants. Since it was not possible to do so, the following experiment was devised to overcome this.

The standard procedure followed in the isolation of fimbriate transductants was carried out in duplicate, with phage and broth controls included as usual. The known good recipient Sa 706 and lysate prepared on Sa 497 were used. At the end of the first 48 hr. both tubes were subcultured as normally. The first 48 hr. broths were then homogenised and samples removed to estimate their turbidity. Serial ten-fold dilutions in broth were made from the first 48 hr. homogenised broth, and three chosen dilutions plated out to give 10-1000 colonies per 0.1 ml.,

(a) on giant (16 cm.) plates containing 100 ml. of phosphate buffered agar per plate, and incubated for two days at 37°. This medium has been found satisfactory for testing directly for haemagglutinating activity in Salmonella strains without nutrient broth subculture (Duguid, personal communication). Fifty such giant colonies were tested for each dilution, and colonies found to be non-fimbriate by this method were confirmed by subsequent broth subculture.,

(b) the same three dilutions were plated out on rhamnose and inositol EMB solid medium to detect any fermenting colonies.,

(c) three dilutions giving 10^3-10^5 cells per 0.1 ml.

Table 12. Calculation of cotransduction rates for the gene *fim* with *rha* or *inl* in *Salmonella typhimurium* recipient FIRN strains.

Test no.	Serial (48 hr.) culture.	Turbidity E 530 mμ	H.P.	% fimbriate	%*fim*+*rha*+	%*fim*+*inl*+
				cells in population.		
1.	1st	0.648	300	58	<0.001	<0.001
	2nd	0.748	1000	92	<0.001	<0.001
2.	1st	0.642	250	48	<0.001	<0.001
	2nd	0.668	1200	94	<0.001	<0.001

Recipient strain *S.typhimurium* Sa 706 was treated with transducing lysate from *S.typhimurium* Sa 497, and *fim*+ recombinants selected. Homogenised broths at 48 hr. intervals were diluted in broth, and, dilutions calculated to give 10 - 1000 colonies per 0.1 ml. plated (I) on phosphate-buffered agar for direct testing of single colonies for fimbriation, and (II) on rhamnose and inositol EMB agar to detect any fermenting colonies. Dilutions, giving c. 10^3 - 10^5 cells per 0.1 ml. were plated on rhamnose and inositol minimal media to detect *rha*+ or *inl*+ cotransductants. Rhamnose or inositol utilising colonies were never detected either on EMB or minimal agars.

were plated on inositol and rhamnose minimal media.

The same procedure was followed through at the second 48 hr. broth subculture. Haemagglutinating powers were estimated (Duguid and Gillies, 1957), and control plates of all media checked with strains of known behaviour. The accompanying table shows that there was a large number of fimbriate cells at both first and second broths, but no rhamnose or inositol-fermenting cells detected. (table 12).

Flagellation.

The non-fimbriate Dubnau strains, Sa 1134 and 1137, were, because of their non-motility, of no value as recipients in transduction to fimbriation. Before the motile recombinant of these strains (Dubnau 18) was obtained from Miss Sylvia Smith, attempts were made to transduce motility into this Sa 1134-1137 line with a range of FIRP and FIRN flagellate donors, using the semi-solid gelatin selective medium of Stocker et al (1953). This was successful, and was repeated using another isolation method. After allowing time for the adsorption of phage (propagated on S.typhimurium FIRP strain 497), the 10 ml. volume of phage-bacterium mixture was dispensed in small volumes, c.0.I ml., into 75-100 Craigie tubes with 0.4% semi-solid agar. Controls showed that motile mutants did not arise spontaneously. Flagellate, motile transductants, swimming through the agar from their point of inoculum, were picked into nutrient broths. After 48 hr. incubation, each of these broths was spun and their deposits tested. A few were found to

be haemagglutinating, i.e. cotransduction of *fim* and *fla* was apparently occurring at a low frequency. Results suggested that the maximum cotransduction rate was 4%. The incidence of *fim*+ among *fla*+ transductants was probably much less than this figure, since the inoculum (into broth for testing of fimbriation) of phage-treated bacteria in the soft agar was a mass inoculum of *fla*+ cells, and not single colonies. A possible explanation of these findings, not involving the occurrence of cotransduction, is simply that transduction of *fla*+ made possible the selective outgrowth of spontaneous fimbriate revertants. However, phage propagated on a motile, non-lysogenic derivative of the Sa 1134-1137 line (TV 180), although an efficient donor of flagellation to the Sa 1134-1137 group, gave no class of fimbriate or flagellate transductants. (This strain TV 180 is a mutant from the same LT-2 line as Sa 1137, derived by ethyl methane sulphonate treatment, transduced to motility, but non-lysogenic, and given col E_I - obtained by Dr. B.A.D. Stocker).

Although fimbriate recombinants had been detected in crosses between Dubnau and FIRN strains, no cotransduction of *fim* and *fla* was detected when lysates from FIRN strains were used as donors in the above experiments with Sa 1134 or 1137 as recipient. Theoretically, such a class of *fim*-*fla* cotransductants should have been detected, but, perhaps, this occurred at a rate too low to be calculated. Unfortunately, this phenomenon of apparent cotransduction

appeared late in these investigations and a detailed analysis was not possible.

Treatment of fimbriate Salmonella typhimurium strains with acriflavine.

It is a useful criterion of episomes that cells which carry them can be suitably disinfected by acriflavine when the episome is carried cytoplasmically (Hirota and Iijima, 1957, Watanabe and Fukasawa, 1961). These findings for the F+ factor in E.coli and resistance transfer factor in Shigella were not applicable to the fertility episome in Pseudomonas (Holloway and Fargie, 1960).

Salmonella typhimurium Sa375, a typical phase-varying strain, was chosen for the investigation of acriflavine exposure and its ability to disinfect fimbriate cells. This strain was serially subcultured through two 48 hr. nutrient broths until the approximate percentage of fimbriate cells was 95 - 100%. This fully-fimbriate strain was subsequently subcultured through acriflavine broths (20 µg./ml.) in like manner, without any observable decrease in the percentage of fimbriate cells, although the final cell population per subculture was, naturally, lower. This suggests that the hypothetical episome is either acriflavine resistant or that expression of fimbriation by serial broth subculture depends on an integrated, chromosomal episome. If the latter hypothesis were correct, then the environmental suppression of fimbriation would be equated to the episome's change from the integrated to the autonomous state.

To test this theory, the same fimbriate strain Sa 375

was subcultured through nutrient agar and acriflavine agar (20μg. and 50 μg./ml.) in parallel. Duguid and Wilkinson (1961) showed that for Shigella flexneri strains even one such subculture was capable of reducing the percentage of fimbriate cells by 50%. After acriflavine exposure on agar, single colonies were picked into nutrient broth and tested after 24 hr. at 37° for haemagglutinating activity. The percentage number of cells did not differ from that of a control culture not exposed to acriflavine.

Attempts to transfer fimbriation from Salmonella typhimurium FIRP to FIRN strains by contact experiments.

The absence of an acridine-resistant episome in Salmonella typhimurium FIRP strains was indicated by the following procedure. A series of streptomycin-resistant mutants was isolated by exposing them to increasing concentrations of streptomycin, until a mutant of Sa 7471 was isolated, resistant to 1000 μg./ml. of streptomycin. An overnight broth culture of the non-lysogenic FIRP strain Sa 206 was diluted I:10 in nutrient broth to give a final cell concentration of 5×10^8 per ml. 2.5 ml. of this diluted culture and 1.5 ml. of Sa 7471 (str-r) were incubated with gentle aeration in mixed culture in nutrient broth for times up to three hours after mixing. At chosen time intervals, 4 ml. of streptomycin broth (2000μg./ml.) were added to each test tube to kill the streptomycin-sensitive fimbriate donor. Incubation was then allowed to proceed in aerobic, static broths for 48 hr. before subculture into normal nutrient broth. Controls showed that the fimbriate

Table 13. Transduction of haemagglutinating ability to fimbriate, but non-haemagglutinating, *Salmonella paratyphi* B strains.

Recipient strain: Sa reference no.	Phage type	No. of tests giving transduction to *Ha* + / no. of tests performed with phage prepared on donor strain that was:								No. of tests giving mutation to *Ha* + / no. of tests made without added phage.
		Fimbriate				Group A	FIRN			
		497	1451	1541	1566a	1436	612	1292	1300	
66		11/51	0/4	1/8	0/4	0/4	0/4	0/4	0/4	0/17
964	Taunton	5/8	.	.	.	.	.	.	.	0/1
1309	3A	0/19	.	.	.	.	.	.	.	0/2
1313	3B	4/8	.	.	.	.	.	.	.	0/1
1361	3B	2/8	.	.	.	.	.	.	.	0/1
1363	Taunton	1/19	.	.	.	.	.	.	.	0/2

donor Sa 206 did not produce a one-step streptomycin-resistant (1000 µg./ml.) mutant. Haemagglutination was detected in some first 48 hr. deposits but was due to residual haemagglutination from the donor, since all second 48 hr. normal broths were sterile.

Experiments with non-haemagglutinating fimbriated strains.

Electron microscopy has revealed a number of Salmonella paratyphi B strains which are typically fimbriate but not capable of haemagglutinating red cells (J.P. Duguid, personal communication). Six such strains, representing three different phage types, were chosen as recipients in transduction experiments. Transducing lysates were prepared on both fimbriate and non-fimbriate strains of Salmonella typhimurium and on certain of the S.paratyphi strains. Broth subculture showed that these fimbriated, non-haemagglutinating strains were unable to produce the pellicles characteristic of normally fimbriate strains. (Old, unpublished results). It was reasoned that serial broth subculture, and the pellicle formation which allows successful out-growth of haemagglutinating cells, would prove a satisfactory selective medium.

The accompanyingtable/13 shows the results of these experiments. Of the six strains used - Sa 66, 964, 1309, 1313, 1361 and 1363 - only Sa 1309 was not transduced to haemagglutinating ability with lysates from fimbriate S.typhimurium strains. Transduction to haemagglutinating ability was not observed with lysates prepared on the non-

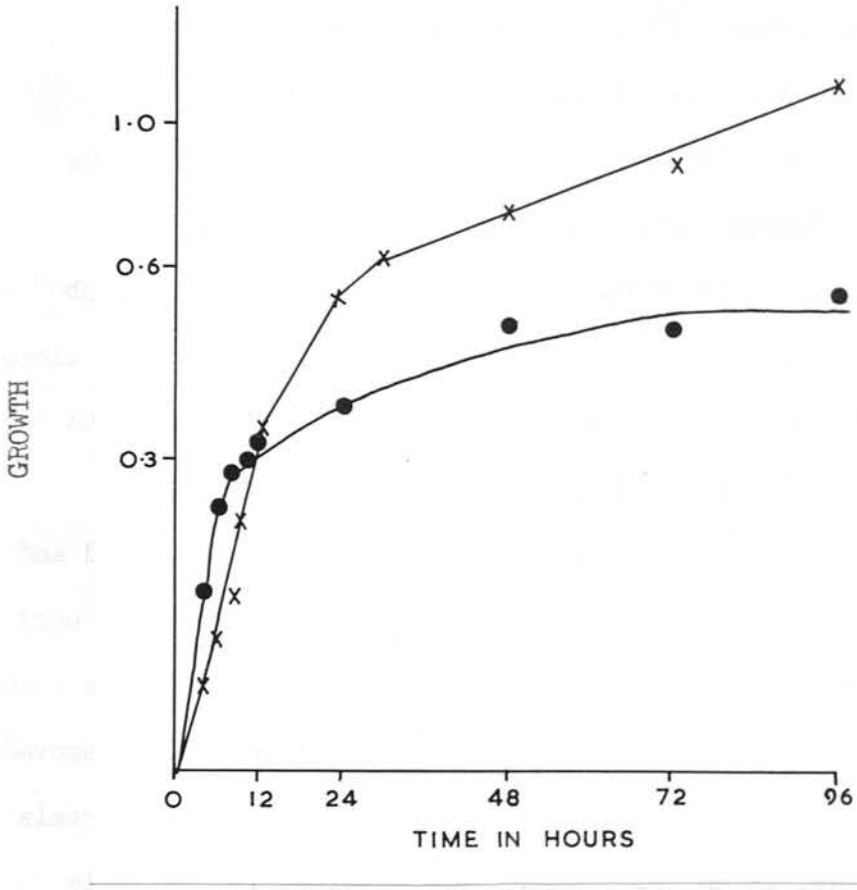


Fig.2. Amount of growth of Salmonella paratyphi B, in phosphate-buffered broth, incubated aerobically and statically at 37°. x----x strain Sa66 Ha+ is a fimbriate, haemagglutinating transductant. ●---● strain Sa66 Ha- is fimbriate and non-haemagglutinating. Turbidity readings are plotted logarithmically. Inocula, giving c. 10^7 bacilli/ml., were taken from broth culture after successive subcultures of 48 hr. and 28 hr. A pellicle became visible with Sa 66 Ha + after 8 hr.

fimbriate FIRN and group A strains, suggesting that these too lack this gene. Lysates from other S.paratyphi strains of the above group failed to give recombination with any other strain as recipient.

Parallel growth experiments on the two kinds of fimbriated S.paratyphi B, the parent and its transductant, were approached with the intention of proving that the latter reached greater growth levels in nutrient broth under aerobic and static conditions, attributable to its pellicle-forming ability. The two strains used were S.paratyphi B, Sa 66 Ha+ and Sa 66 Ha-. These were sub-cultured from their preservation slopes (single colony lines) onto nutrient agar for 24 hr. and then in nutrient broth for periods of 48 and 24 hr., incubating aerobically and statically. The latter 24 hr. broths were homogenised and diluted in broth for counting. Single 0.02 ml. drops were pipetted into large numbers of 10 ml. broths for each strain. Duplicate tubes of both Sa 66 Ha+ and Sa 66 Ha- were removed at two hour intervals up to 12 hr., and at 24 hr. intervals from 24 hr. to 96 hr. These were examined for pellicle formation, formaldehyde added, and turbidities and haemagglutinating powers estimated. The growth curves obtained are represented in the accompanying figure 2.
It can be seen that under identical conditions the strain with the functional fimbriae attains a final level of growth twice that of the strain with inactive fimbriae . This out-growth is concomitant with, and resultant from, the

appearance of a pellicle in Sa 66 Ha+ at 8 hr. The growth potential resulting from such a property is obviously great.

SECTION II

Competitive growth between fimbriate and non-fimbriate strains of Salmonella typhimurium in mixed culture under a range of environmental conditions, when both strains are flagellate.

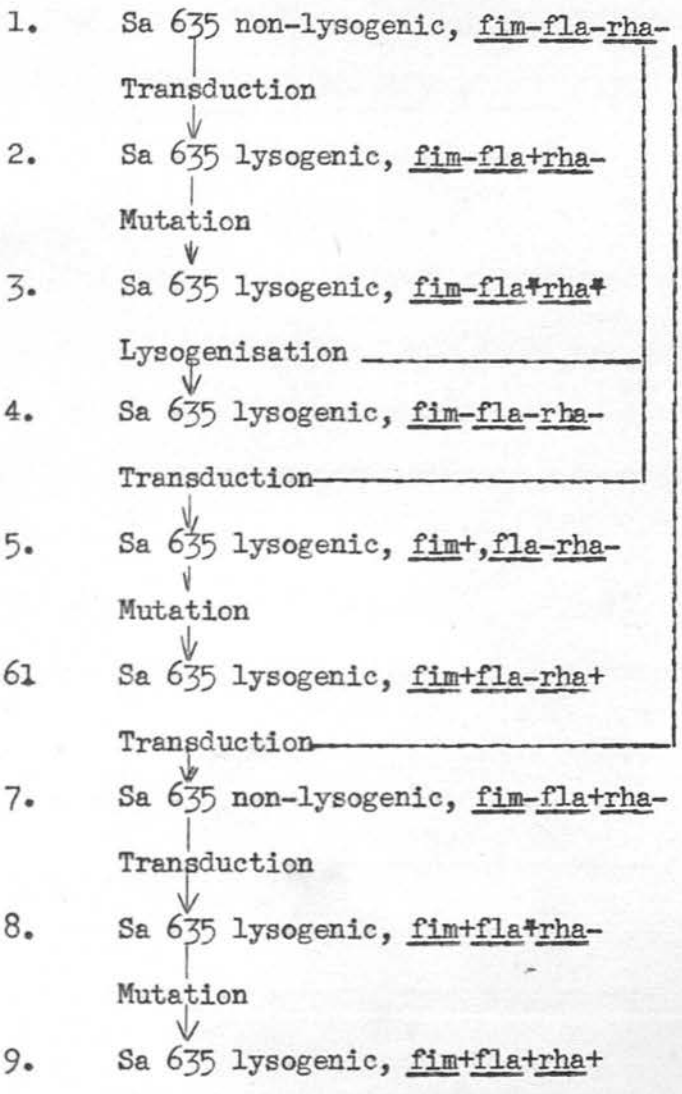
In all competition experiments, the phenomenon of selection of fimbriate cells grown in mixed culture with non-fimbriate cells was examined by the addition of small numbers of fimbriate cells to large numbers of non-fimbriate cells. Each fimbriate cell was also rhamnose-fermenting, either naturally or by transduction or by selection of mutants, so that this marker could be used for differential counting. All strains used were Salmonella typhimurium. There were three classes:

(i) naturally-isolated strains of FIRP and FIRN status, the former class represented by S.typhimurium-LT2 and Sa 206, the latter by S.typhimurium Sa 7471. All three are non-lysogenic and there was no evidence of colicin activity between Sa 7471 and either of the two FIRP strains.

(ii) S.typhimurium Sa 625 was transduced first to fimbriation and then to strong rhamnose fermentation for use as challenger fimbriate organism. The lysogenic Sa 625 fim- rha- isolated from the transduction mixture was used as challenged organism.

(iii) S.typhimurium Sa 635 is a non-lysogenic, non-flagellate, non-fimbriate FIRN strain. From it were isolated flagellate and fimbriate lysogenic transductants, and from each a rhamnose-strong-fermenting mutant selected by plating out on rhamnose minimal agar. From the non-lysogenic, flagellate transductant of Sa 635 (i.e. Sa 1183)

a fimbriate transductant was isolated, from which a rhamnose-strong-fermenting mutant was selected. The origin of all these Sa 635 derivatives is shown in the accompanying diagram.



In the first three experiments to be described, the strains used were the natural isolates, S.typhimurium-LT2 and Sa 7471.

Experiment 1.

Single-colony lines of these two strains were subcultured from preservation slopes on to nutrient agar for

Table 14. Competitive growth in mixed culture between naturally isolated fimbriate and non-fimbriate strains of *Salmonella typhimurium*, both strains being flagellate.

F = strain LT_2 *fim+ fla+ rha+* v. N = strain Sa 7471 *fim- fla+ rha-*

Cultural conditions	Challenger mixture	Time in hr.	Turbidity E 530	Pellicle	Counts x 10^6/ml.		% fimbriate cells in population	H.P.
					Viable	Fimbriate		
Aerobic static broth	LF + N	0	0.116		.	0.000005	0.000016	
		6	0.302	-	320	<0,001	0.0003	0
		24	0.389	R+	610	0.5	0.08	2
		48	0.518	"++++"	520	36	7.	2
	HF + N	0	0.116		.	0.0052	0.016	
		6	0.302	-	330	0.077	0.023	0
		24	0.394	R+	470	52	11	20
		48	0.446	+++	400	93	23	20
Micro-aerophilic, static broth.	LF + N	0	0.116		.	0.000005	0.000016	
		6	0.284	-	320	<0.001	0.003	0
		24	0.335	-	355	0.5	0.14	0
		48	0.346	+++	450	77	17	400
	HF+N	0	0.116		.	0.0052	0.016	
		6	0.290	-	310	0.050	0.016	0
		24	0.338	-	365	31	8.5	20
		48	0.355	++	375	137	42	600

Challenger doses of LT2 were LF = 52 organisms or 5.2/ml.
HF = 52,000 " or 5.2×10^3/ml.

		LF Challenger control.			HF Challenger control.			N Challenged control	
Cultural conditions	Time in hr.	Turbidity E 530	Pellicle	H.P.	Turbidity E 530	Pellicle	H.P.	Turbidity E 530	Pellicle
Aerobic, static broth.	0	0.000			0.000			0.116	
	6	0.038	-	0	0.200	-	0	0.296	-
	24	0.391	++	200	0.416	++	300	0.634	+++
	48	0.542	+++	500	0.610	"+++"	250	0.429	++
Micro-aerophilic static broth.	0	0.000			0.000			0.116	
	6	0.040	-	0	0.164	-	0	0.268	-
	24	0.316	-	20	0.332	+++	400	0.325	-
	48	0.392	+++	800	0.444	+++	800	0.330	-

In all competition experiments the following abbreviations are used.
R = rim of growth on glass above broth surface; "-" = granular, flagellar pellicle present.
H.P. = haemagglutinating power.

24 hr. and into nutrient broth for 24 hr. At the time of challenge, the viable count of the Sa 7471 (N) cells was 32 x 10^6 organisms per ml., and the challenger doses were, respectively, 52 (LF) organisms and 52 x 10^3 (HF) organisms. In this experiment, the broths were incubated statically and aerobically and microaerophilically. Table 14 shows the results of this experiment. Under aerobic conditions, both the low level (LF) and high level (HF) challenger doses outgrew the challenged non-fimbriate cells, giving, respectively, at 48 hr. final fimbriate populations of 7% and 23%. Under microaerophilic conditions, the final growth levels were less, but the low and high challenger levels had competed even more successfully, giving final fimbriate populations of 16.9% and 42%. The two strains differ in the following characters:

		LT2 Fimbriate	Sa7471 Non-fimbriate
Fermentation of	Rhamnose	Strong and early	Late
	Xylose	Strong and early	Weak and early
	Inositol	Strong and early	Negative
	Trehalose	Late	Strong and early
Gas production		Anaerogenic	Aerogenic

Thirty-four red and thirty-four pale colonies were picked at random from a selection of rhamnose EMB plates and tested. All 34 red colonies behaved as typical S.typhimurium-LT2 strains, and all 34 pale colonies were typically Sa 7471. No hybrids with mixed characters were observed.

Experiment 2.

In this experiment, the low and high levels of challenger strain LT2 were, respectively, 130 and

Table 15. Competitive growth in mixed culture between naturally isolated fimbriate and non-fimbriate strains of *Salmonella typhimurium*, both strains being flagellate.

F = strain LT2 *fim+fla+rha+* v. N = strain Sa 7471 *fim-fla+rha-*

Cultural conditions	Challenger mixture	Time in hr.	Turbidity E 530	Pellicle	Counts x 10^6/ml.		% fimbriate cells in population.	H.P.
					Viable	Fimbriate		
Micro-aerophilic, static broth	LF+N	0	0.187		170	0.000013	0.000006	
		6	0.364	-	360	0.0001	0.000003	0
		24	0.454	+++	315	0.28	0.089	0
		48	0.424	+++	135	22	16	50
	HF+N	0	0.187		170	0.013	0.006	
		6	0.349	-	435	0.121	0.028	0
		24	0.470	+++	350	25	7.1	50
		48	0.446	+++	90	24	26.0	100
Anaerobic, static broth.	LF+N	0	0.187		170	0.000013	0.000006	
		48	0.247	-	99	0.77	0.77	0
	HF+N	0	0.187		170	0.013	0.006	
		48	0.266	-	87	4.6	5.7	0
,Rotated broth.	LF+N	0	0.187		170	0.000013	0.000006	
		6	0.795		1000	0.0005	0.00005	0
		24	0.972		1650	0.0035	0.00021	0
		48	0.996		1300	0.48	0.038	0
	HF+N	0	0.187		170	0.013	0.006	
		6	0.819		1410	0.42	0.030	0
		24	0.930		2000	2.4	0.120	0
		48	0.987		900	10	1.1	0

		LF Challenger control.			HF Challenger control.			N Challenged control.	
Cultural conditions	Time in hr.	Turbidity E530	Pellicle	H.P.	Turbidity E 530	Pellicle	H.P.	Turbidity	Pellicle
Micro-aerophilic, static broth.	0	0.000			0.000			0.187	
	6	0.018	-	0	0.201	-	0	0.344	-
	24	0.560	+++	500	0.600	+++	500	0.470	-
	48	0.440	+++	500	0.592	+++	600	0.446	++
			pH						pH
Rotated broth.	0	0.000	7.0					0.187	7.0
	6	0.009	.	0				0.741	.
	24	0.600	.	1				0.888	.
	48	0.762	7.2	1				0.996	7.2
Anaerobic broth.	0	0.000			0.000				
	48	0.278	-	20.	0.266	-	20	0.264	-

Challenger doses of LT2 were LF = 130 organisms.
HF = 130,000 organisms.
At time of challenge , N = 170 x 10^6/ml.

130×10^3 organisms. At the time of challenge, the viable counts of the challenged non-fimbriate strain, Sa 7471, was 170×10^6 organisms per ml. Under microaerophilic conditions, both low and high challenger levels reached significantly high final populations of 16 and 26%, respectively. In broths incubated anaerobically, with a high challenger dose, 5.7% of the final population was fimbriate, although no haemagglutinating activity was detectable, and no pellicle was formed. No pellicle was formed in broths continually rotated at a speed of 12 revolutions per minute, and yet the high challenger dose of 130×10^3 organisms yielded a final 48 hr. population of 1.1% fimbriate cells. The increase in the number of fimbriate cells under microaerophilic conditions, was accompanied by an increase in the haemagglutinating power of the broth from 0-100, with the onset of pellicle formation. Twenty-four red and twenty-four pale colonies were again randomly chosen and tested for fimbriation status and for the fermentation of the four sugars. All 48 colonies behaved correctly, either as typical S.typhimurium strains of LT2 or Sa 7471 origin. (see table 15)

Experiment 3.

In the final experiment with these two strains, phosphate buffered broths and agars were used, and the competition experiments performed aerobically and statically, anaerobically, in shaken flask cultures on a reciprocating shaker and on agar plates. Low and high challenger doses were used for all conditions, except the plates in which

Table 16. Competitive growth in mixed culture between naturally isolated fimbriate and non-fimbriate strains of Salmonella typhimurium, both being flagellate.

F = strain LT2 fim+fla+rha+ v. N = strain Sa 7471 fim-fla+rha-

Cultural conditions	Challenger mixture	Time in hr.	Turbidity E 530	Pellicle	Counts x 10^6/ml.		% fimbriate cells in population.	H.P.
					Viable	Fimbriate		
Aerobic, static broth.	LF+N	0	0.097		190	0.0000012	0.0000006	
		6	0.324	-	385	0.00001	0.000002	0
		24	0.488	-	400	0.0001	0.000002	0
		48	0.514	++	340	12	3.6	30
	HF+N	0	0.097		190	0.0012	0.006	
		6	0.336	-	410	0.005	0.0012	0
		24	0.442	+	555	0.90	0.16	2
		48	0.574	++	360	13	3.6	50
Anaerobic, static broth	LF+N	0	0.097		190	0.0000012	0.0000006	
		48	0.239	-	42	-	0.000025	0
	HF+N	0	0.097		190	0.0012	0.006	
		48	0.249	-	45	1.2	2.7	0
Artifically shaken broth.	LF+N	0	0.097	pH	190	0.0000012	0.0000006	
		6	2.680		3300	0.003	0.000009	0
		24	2.568	7.45	3600	0.14	0.0031	0
		48	2.896	7.40	2500	0.50	0.014	0
	HF+N	0	0.097		190	0.0012	0.0006	
		6	2.440		2700	0.31	0.011	0
		24	2.432	7.44	6700	23	0.34	0
		48	2.816	7.36	3000	21	0.70	0
Plates.	HF+N	0	0.097		190	0.0012	0.0006	
		6	3.120	7.5	3800	0.164	0.0043	0
		24	4.008	7.5	9600	1.4	0.014	0
		48	4.008	7.5	3500	50	1.41	0

		LF Challenger control.			HF Challenger control.			N.Challenged control.	
Cultural conditions.	Time in hr	Turbidity E 530	Pellicle	H.P.	Turbidity E 530	Pellicle	H.P.	Turbidity E 530	Pellicle
Aerobic, static broth.	0	0.000			0.000			0.097	
	6	0.000	-	0	0.109	-	0	0.317	-
	24	0.348	-	25	0.382	-	25	0.426	-
	48	0.792	+++	1000	1.084	"++++"	1000	0.520	++
Shaken	0	0.000	7.0 pH		0.000	7.00 pH		0.097	pH 7.00
	6	0.000	7.00	0	0.284	7.00	0	2.424	7.40
	24	2.328	7.30	1	2.224	7.32	2	2.528	7.45
	48	1.824	7.24	25	2.528	7.18	10	2.896	7.34
Plates.	0				0.000	7.00		0.097	7.00
	6				0.026	7.00	0	2.944	7.50
	24				3.072	7.42	25	4.120	7.54
	48				3.200	7.36	50	4.080	7.44
Anaerobic static broth.	0	0.000	.	.	0.000	.	.	0.097	.
	48	0.227	-	3	0.236	-	2	0.216	-

LF challenger dose = 12 organisms. HF challenger dose = 12 x 10^3 organisms.

only the high challenger dose was tested. Counts were performed on the anaerobic broths only at 48 hr. The results are expressed in table 16. A total fimbriate population of 3.6% was reached by both low and high challenger levels at 48 hr., when the competition proceeded aerobically and statically. This was accompanied by the formation of a slight pellicle and by an increase in the haemagglutinating power. The high challenger dose of 12,000 organisms showed final fimbriate populations of 2.7%, 1.41% and 0.7% when incubated, respectively, under anaerobic, plate and shaken conditions, i.e. only slightly less than that reached when incubated aerobically and statically. These tests, however, unlike the aerobic-static test, lacked haemagglutinating activity. Twenty red and twenty pale colonies were, again, selected and characterised as either S.typhimurium LT2 or Sa 7471.

Experiment 4.

In this, the final competition experiment with naturally-isolated strains of S.typhimurium, the fimbriate strain used was FIRP, Sa 206. Sa 7471 was, again, the non-fimbriate strain used as challenged organism. The competition was carried out under aerobic and static, anaerobic and static conditions, and also artificially aerated on a reciprocating shaker. At the time of challenge, the viable count of the non-fimbriate strain was 42×10^6 organisms per ml., and the low and high levels of fimbriate Sa 206 were 3 and 300 organisms,

Table 17. Competitive growth in mixed culture between naturally isolated fimbriate and non-fimbriate strains of S.typhimurium, both strains being flagellate.

Sa 206 fim+ fla+ rha+ v. Sa 7471 fim- fla+ rha-

Cultural conditions	Challenger mixture	Time in nr.	Turbidity E530	Pellicle	Viable count x 10^6/ml.	% fimbriate cells in population	Haemagglutinating activity in broth/deposit
Aerobic, static broth	LF+N	0	0.087		42	•	
		24	0.456	-	500	0	-/-
		48	1.500	+++	980	10	+++/+++
	HF+N	0	0.087		42	•	
		6	0.379	-	330	0	-/-
		24	0.490	++	700	8	-/-
		48	1.530	+++	1200	17	+++/+++
Anaerobic, static broth	LF+N	0	0.087		42	•	
		24	0.326	-	380	0	-/-
		48	0.294	-	70	0	-/-
	HF+N	0	0.087		42	•	
		6	0.354	-	470	0	-/-
		24	0.357	-	310	0	-/-
		48	0.280	-	70	0	-/-
Artificially aerated by shaking	LF+N	0	0.087		42		
		24	3.280	-	3600	0	-/-
		48	3.886	-	120	0	-/-
	HF+N	0	0.087		42		
		6	4.840	-	6200	0	-/-
		24	3.420	-	1200	0	-/-
		48	3.820	-	1200	3.5	-/-

		HF Challenger control		N Challenged control	
Cultural conditions	Time in hr.	Turbidity E 530	Pellicle	Turbidity E 530	Pellicle
Aerobic, static.	0	0.000		0.087	
	6	0.025	-	0.368	-
	24	0.425	P	0.374	-
	48	1.580	P	0.890	-
Anaerobic, static.	0	0.000		0.087	
	6	0.055	-	0.327	-
	24	0.265	-	0.340	-
	48	0.192	-	0.223	-
Aerated by	0	0.000		0.087	
	6	0.050	-	4.620	-
	24	3.840	-	3.420	-
	48	3.750	-	3.500	-

LF challenger dose was 3 organisms.
HF challenger dose was 300 organisms.

respectively. Table 17 shows that both low and high challenger levels of Sa 206 had reached significant final percentages of the total population under aerobic and static conditions. It is also seen that, under shaken conditions, the high challenger level reached a final fimbriate population of 3.5%, although the cells were not phenotypically fimbriate.

This concluded the experiments with naturally-isolated FIRP and FIRN strains of S.typhimurium. It is seen that under conditions favourable to the formation of a fimbrial pellicle, viz. aerobic and microaerophilic static broths, a considerable selection of the fimbriate cells occurred. However, non-specific outgrowth of both Sa 206 and LT-2 against Sa 7471 was observed under conditions in which the outgrowth was not accompanied by an increase in the haemagglutination power of the cultures. In the experiments outlined to date, unrecognised strain differences in the natural isolates other than fimbriation might have favoured one strain, and so account for the non-specific outgrowth under conditions where pellicle-formation played no part. To overcome this defect, transductant fimbriate and non-fimbriate pairs from a single parent, isolated from the same transduction mixture, were used in all further experiments. Because transduction is so limited in its transfer of genetic material, it is correct to assume that the only difference in such challenger and challenged strains will be due to the marker transduced, viz. fimbriation.

Table 18. Competitive growth in mixed culture between fimbriate and non-fimbriate strains of *Salmonella typhimurium*, both being flagellate.

F = Sa 625 *fim+fla+rha+* v. N = Sa 625 *fim-fla+rha-*

Cultural conditions	Challenger mixture	Time in hr.	Turbidity E 530	Pellicle	Counts x 10^6/ml. Viable	Counts x 10^6/ml. Fimbriate	% fimbriate cells in population	H.P.
Aerobic, static broth.	LF+N	0	0.136		40	0.0000014	0.0000035	
		6	0.372	-	350	0.00011	0.000031	0
		24	0.516	++	520	17.5	3.4	2
		48	1.880	"++++"	1910	1650	82	200
	HF+N	0	0.136		40	0.0014	0.0035	
		6	0.386	-	395	0.0320	0.008	0
		24	0.920	"++++"	2100	1425	29.0	200
		48	1.880	"++++"	1720	1530	86.0	200
Anaerobic, static broth.	LF+N	0	0.136		40	0.0000014	0.0000035	
		48	0.289	-	75	0.00003	0.000029	0
	HF+N	0	0.136		40	0.0014	0.0035	
		48	0.289	-	134	0.0042	0.0031	0
Artificially shaken broth.	LF+N	0	0.136	pH	40	0.0000014	0.0000035	
		6	3.208	6.9	5750	0.0002	0.0000034	0
		24	3.664	6.9	8700	0.0001	0.0000011	0
		48	3.760	6.9	3700	0.0000	-	0
	HF+N	0	0.136		40	0.0014	0.0035	
		6	3.848	6.9	5600	0.110	0.0020	0
		24	3.880	6.9	10,700	0.101	0.0094	0
		48	4.000	7.0	3600	0.070	0.0018	0
Plates.	HF+N	0	0.136		40	0.0014	0.0035	
		6	3.340	7.50	3300	0.140	0.0042	0
		24	4.700	7.45	15000	0.270	0.0018	0
		48	5.400	7.44	7000	0.260	0.0037	0

Challenger doses were LF = 14 organisms.
HF = 14 x 10^3 organisms.
All media was buffered at pH 7.0

		LF Challenger control.			HF Challenger control.			N Challenged control.	
Cultural conditions	Time in hr.	Turbidity E 530	Pellicle	H.P.	Turbidity E 530	Pellicle	H.P.	Turbidity E 530	Pellicle.
Aerobic, static broth.	0	0.000			0.000			0.136	
	6	0.056	-	0	0.114	-	0	0.362	-
	24	0.528	"++++"	20	0.764	++	5	0.492	-
	48	1.880	"++++"	200	1.880	"++++"	200	0.636	++
Artificially shaken broth.	0	0.000			0.000			0.136	
	6	0.082		0	0.544		0	3.400	
	24	3.648		5	3.600		5	3.424	
	48	3.840		2	3.752		2	3.520	
, Plates.	0				0.000			0.136	
	6				0.780		0	3.580	
	24				4.940		25	4.680	
	48				5.000		25	4.680	
Anaerobic static.	0	0.000	.	.	0.000	.	.	0.136	
	48	0.259	-	10	0.272	-	10	0.305	-

Experiment 5.

In this experiment, the fimbriate and non-fimbriate lysogenic pair derived from S.typhimurium FIRN, Sa 625, was used. Each strain was also flagellate. Low and high levels of the fimbriate challenger, F = Sa 625 fim+fla+ rha+, 14 and 14 x 10^3 organisms, respectively, were used to challenge the non-fimbriate strain, N = Sa 625 fim-fla+rha-, when it had reached a cell concentration of 40 x 10^6 cells per ml. Buffered media were incubated aerobically and anaerobically, in shaken cultures and on plates. The results in table 18 show the detailed analysis of this experiment. Even at 24 hr. both low and high challenger doses compete successfully against the non-fimbriate strain under aerobic-static conditions, giving considerable final fimbriate cell populations. At 48 hr., this had increased to 82 and 86% final fimbriate populations for low and high challenger levels, and was accompanied in both cases by the formation of fimbrial pellicles and an increase in haemagglutinating power. Mixed cultures of challenger and challenged cells incubated in broth - anaerobically or shaken - or on plates, gave no competitive outgrowth by the fimbriate challenger in the absence of pellicle formation.

Experiment 6.

A second transduction pair was used to show that the high degree of competition in the previous experiment was not strain-specific. The two cell lines employed were challenger, F = Sa 635 fim+fla+rha+ (i.e. Sa 635/9 in

diagram) and challenged, N = Sa 635 fim-fla-rha- (Sa 635/4). Mixed cultures were incubated in buffered broth, aerobically and statically and in shaken culture. A single challenger dose was now used, aimed at a level between the low (LF) and high (HF) challenger doses previously tested. At the time of challenge, the challenger dose was 3,300 organisms, and the viable count of the non-fimbriate challenged strain was 130×10^6 per ml. The formation of a pellicle by the fimbriate strain under aerobic-static conditions enabled it to outgrow its non-fimbriate relative. At 24 and 48 hr., 30% and 46% of the total population was fimbriate (see table 19). This same fimbriate strain, however, failed to outgrow its non-fimbriate partner in mixed culture, if shaken artificially at 100 oscillations per min, and the percentage fimbriate population remained constant throughout the 48 hr. period of mixed growth. This experiment confirms the results of experiment 5 for a second transduction pair. In both experiments 5 and 6, twelve red and twelve pale colonies were randomly selected and tested for their fimbriation status and ability to ferment rhamnose. All fimbriate strains were rhamnose-fermenting, all non-fimbriate strains were rhamnose non-fermenting.

This experiment completed a series of reconstructions in which attempts have been made to simulate the situation present in the commonest transduction-to-fimbriation experiments, in which a flagellate FIRN strain had been

Table 19. Competitive growth in mixed culture between fimbriate and non-fimbriate strains of *Salmonella typhimurium*, both strains being flagellate.

F = Sa 635 *fim+ fla+ rha+* v. N = Sa 635 *fim- fla+ rha-*

Cultural conditions	Time in hr.	Turbidity E. 530.	Pellicle	Counts x 10^6/ml.		% fimbriate cells in population	H.P.
				Viable	Fimbriate		
Aerobic, static broth.	0	0.159		130	0.00033	0.00025	
	6	0.350	-	530	0.00525	0.001	0
	24	0.712	+++	545	165	30	100
	48	1.840	++++	1900	875	46	500
			pH				
Artificially shaken broth	0	0.159	7.0	130	0.00033	0.00025	
	6	3.270	7.1	4200	0.0012	0.00026	0
	24	3.480	7.1	4600	0.0029	0.00025	0
	48	3.610	7.3	4650	0.0093	0.00020	0

		Challenger F control			Challenged N control	
Cultural conditions	Time in hr.	Turbidity E 530	Pellicle	H.P.	Turbidity E 530	Pellicle
Aerobic, static broth.	0	0.000			0.159	
	6	0.071	-	0	0.390	+
	24	0.742	+++	250	0.490	R ++
	48	1.840	++++	300	0.768	R ++
			pH			pH
Artificially Shaken broth.	0	0.000	7.0		0.159	7.0
	6	0.065	7.0	0	3.410	7.1
	24	3.380	7.1	10	3.590	7.1
	48	3.600	7.15	10	3.540	7.2

F challenger dose = 3,300 organisms.

Table 20. Competitive growth in mixed culture in aerobic, static broth between fimbriate and non-fimbriate strains of *Salmonella typhimurium*, both strains being non-flagellate.

F = Sa 635 *fim*+ *fla*- *rha*+ v. N = Sa 635 *fim*- *fla*- *rha*-

Time in hr.	Turbidity E 530	Pellicle	Counts x 10^6/ml.		% fimbriate cells in population	H.P.
			Viable	Fimbriate		
0	0.259		130	0.000215	0.00017	
6	0.395	-	140	0.00005	0.000036	0
24	0.592	R +	170	0.00008	0.00005	0
48	0.572	++	400	0.00008	0.00002	0

	Challenger F control			Challenged N control	
Time in hr.	Turbidity E 530	Pellicle	H.P.	Turbidity E 530	Pellicle
0	0.000			0.259	
6	0.006	-	1	0.358	-
24	0.510	++	50	0.534	-
48	0.750	+++	250	0.560	R ++

F challenger dose = 215 organisms.

N.B. At the time of challenge, 0 hr. in table, the fimbriate strain, Sa 635 *fim* + , had been subcultured through nutrient agar for 24 hr., and nutrient broth for 24 hr., and so was the phenotypically non-fimbriate phase.

employed, i.e. after the addition of phage, large numbers of fim- fla+ cells would be present and grow in mixed culture with small numbers of fim+ fla+ cells (representing, respectively, non-transduced and transduced fimbriate cells).

Further reconstruction experiments were now evolved to discover the relative and interdependent roles of flagella and fimbriae in these experiments. In section I, we have seen that successful transduction to fimbriation had an almost absolute requirement for motility in the recipient cells. For an explanation of this, a reconstruction experiment was devised in which the conditions of a transduction-to-fimbriation experiment involving a non-motile recipient FIRN strain were simulated.

Competitive growth between fimbriate and non-fimbriate strains of Salmonella typhimurium in mixed culture, when both strains are non-flagellate.

Experiment 7.

The challenged strain, Sa 635 fim-fla-rha- (Sa 635/4) was grown to a density of 130 x 10^6 cells per ml. under aerobic, static conditions in buffered broth. A challenger dose of 215 organisms of Sa 635 fim+fla-rha+ (Sa 635/6) was added corresponding to the hypothetical fimbriate transductants arising from phage addition (although, probably, in excess of what is actually found). Between the times of subculture from the preservation slope and the time of challenge, each strain had been subcultured for 24 hr. on nutrient agar and 24 hr. in nutrient broth, so that the fimbriation of the challenger was probably not fully expressed. Table 20 shows that increased viable counts,

Table 21. Competitive growth in mixed culture in aerobic static broth between fimbriate and non-fimbriate strains of *Salmonella typhimurium*, both strains being non-flagellate.

F = Sa635 *fim+ fla- rha+* v. N = Sa635 *fim- fla- rha-*

Time in hr.	Turbidity E 530	Pellicle	Counts x 10^6/ml.		% fimbriate cells in population	H.P.
			Viable	Fimbriate		
0	0.185		65	0.00017	0.00026	
6	0.346	-	270	0.0008	0.0003	0
24	0.459	-	200	4.8	2.4	0
48	0.482	R++	160	74	46.2	0
72	0.656	R++	290	185	63	2
96	1.411	+++	640	450	70	50

	F Challenger control			N Challenged control	
Time in hr.	Turbidity E 530	Pellicle	H.P.	Turbidity E 530	Pellicle
0	0.000			0.185	
6	0.101	-		0.343	-
24	0.430	-	20	0.426	-
48	1.395	R++	125	0.510	-
72	1.047	++	50	0.798	-
96	1.437	+++	125	1.098	-

F Challenger dose = 3,300 organisms

over the 48 hr. period after time of challenge, are due solely to the non-fimbriate cells. Although a slight pellicle was present at 48 hr., no increase in the percentage of fimbriate cells was noted. Thus, the fimbriate cells had not successfully competed in the absence of flagella. As in all other experiments, twelve large and twelve small colonies were picked (this time from rhamnose DM agar) and their identity checked. All large colonies were fimbriate and rhamnose-fermenting (i.e. Sa 635/6) and all small colonies were non-fimbriate and rhamnose non-fermenting (i.e. Sa 635/4).

Experiment 8.

This experiment was a repeat of experiment 7 under the same conditions, except that each of the two strains was subcultured through three 48 hr. broths to select the fimbriate phase of Sa 635/6. The third broth culture gave a strong +++ haemagglutinating activity with the broth. The duration of this experiment was increased to 96 hr. The challenger dose of fimbriate cells was 3,300 of F = Sa 635/6 and the viable count of the challenged strain, N = Sa 635/4, at the time of challenge was 65×10^6 cells per ml. Between 24 and 48 hr., the decrease in the viable counts of the non-fimbriate cells was accompanied by an increase in the number of fimbriate cells, with the development of a fimbrial rim later extending to a full pellicle. At 96 hr., 70% of the total population was fimbriate (see table 21). This experiment, therefore, revealed a gradual outgrowth by the fimbriate cells, particularly in the 24 - 48 hr. period,

whereas, with other successful competition experiments in which the fimbriate challenger was also motile, (see experiments 4 and 6), competition is observed even at 24 hr.

Competitive growth between flagellate and non-flagellate strains of Salmonella typhimurium in mixed cultures, when both strains are non-fimbriate.

In Shigella flexneri, strains, pellicle formation is not dependent solely on the presence of fimbriae. Heavy, granular flagellar pellicles often occur in permanently non-fimbriate strains (Duguid and Gillies, 1957). This is true also for non-fimbriate S.typhimurium strains.

The next two experiments were designed to evaluate the role of flagella in competition experiments in the absence of fimbriae.

Experiment 9.

The design of these experiments was as before. Sa 635/4, fim-fla-rha-, was the challenged N organism , and, at the time of challenge, gave a viable count of 130 x 10^6 cells per ml. The L challenger organism was Sa 635 fim-fla+rha+ (Sa 635/3) at a challenger level of 200 organisms. The mixture was incubated in buffered broth only under aerobic-static conditions. At 48 hr., 0.15% of the total population was flagellate, which seemed a significant increase, although somewhat smaller than that achieved by fimbriate cells. This outgrowth was accompanied by the formation of a rim around the glass of the tubes at the broth-air interface, and not by an extensive flagellar pellicle. (See

Table 22. Competitive growth in mixed culture in aerobic, static broth between flagellate and non-flagellate strains of *Salmonella typhimurium*, both strains being non-fimbriate.

L = Sa 635 *fim*- *fla*+ *rha*+ v. N = Sa 635 *fim*- *fla*- *rha*-

Time in hr.	Turbidity E 530	Pellicle	Counts x 10^6/ml.		% flagellate cells in population
			Viable	Flagellate	
0	0.259		130	0.00010	0.000076
6	0.420	-	140	0.00007	0.000051
24	0.606	-	300	0.00017	0.000057
48	0.588	R ++	340	0.5	0.147

	Challenger L control		Challenged N control	
Time in hr.	Turbidity E 530	Pellicle	Turbidity E 530	Pellicle
0	0.000		0.259	
6	0.006	-	0.358	-
24	0.464	-	0.534	-
48	0.496	++	0.560	R ++

L Challenger dose = 200 organisms.

Table 23 Competitive growth in mixed culture between flagellate and non-flagellate strains of *Salmonella typhimurium*, both strains being non-fimbriate.

L = Sa 635 *fla+ fim- rha+* v. N = Sa 635 *fla- fim- rha-*

Cultural conditions	Time in hr.	Turbidity E 530mμ	Pellicle	Counts x 10^6/ml.		% flagellate cells in population
				Viable	Flagellate	
Aerobic, static broth	0	0.145		94	0.00023	0.00024
	6	0.375	-	380	0.0008	0.00021
	24	0.552	R 1	495	0.0016	0.00032
	48	0.692	"+++"	540	4.5	0.83
			pH			
Shaken broth	0	0.145		94	0.00023	0.00024
	6	2.910	7.46	6900	0.016	0.00023
	24	3.000	7.56	3800	0.011	0.00030
	48	2.940	7.60	4900	0.011	0.00022

		Challenger L control		Challenged N control.	
Cultural conditions	Time in hr.	Turbidity E 530	Pellicle	Turbidity E 530	Pellicle
Aerobic, static broth	0	0.000		0.145	
	6	0.096	-	0.341	-
	24	0.531	"++"	0.549	R +
	48	1.020	"++++"	0.642	R ++
			pH		pH
Shaken broth.	0	0.000	7.00	0.145	7.00
	6	0.127	7.00	3.000	7.40
	24	2.880	7.50	2.840	7.54
	48	2.990	7.62	3.200	7.64

"P" = granular pellicle

L challenger dose = 2,300 organisms.

table 22).

Experiment 10.

Using the same two strains, the experiment was repeated with a slightly larger challenger dose of flagellate cells, 2300 organisms. This experiment included a control test under shaken conditions (100 oscillations per min.), which would prevent the formation of a flagellar pellicle. At the time of challenge, the viable count of non-flagellate cells was 94 x 10^6 cells per ml. Under aerobic, static conditions, a thick granular +++ pellicle was formed, and at 48 hr. 0.83% of the total population was flagellate (see table 23). The relative increase in numbers of flagellate cells, when incubated in shaken broths, was insignificant, and outgrowth did not occur.

Competitive growth in mixed culture between fimbriate, non-flagellate and non-fimbriate, flagellate strains of Salmonella typhimurium.

Experiment 11.

Sa 635 fim+fla-rha+ (Sa 635/6), at a challenger dose of 3,200 organisms, was used against Sa 635 fim-fla+rha- (Sa 635/2) as challenged organism. At the time of challenge, the viable count of the challenged organism was 190 x 10^6 cells per ml. Each strain was subcultured through two broth subcultures before use as challenger and challenged organism, so that fimbriation in the former was expressed. Nutrient broth (A) was used under only aerobic-static experimental conditions. At 24 hr., neither a fimbrial nor a flagellar pellicle was seen in mixed culture and the total of fimbriate cells in the 24 hr. population was small.

Table 24. Competitive growth in mixed culture in aerobic, static broth between flagellate, non-fimbriate and fimbriate, non-flagellate strains of *Salmonella typhimurium*.

F = Sa635 *fim*+ *fla*- *rha*+ v. N = Sa635 *fim*- *fla*+ *rha*-

Time in hr.	Turbidity E 530	Pellicle	Counts x 10^6/ml.		% fimbriate cells in population	H.P.
			Viable	Fimbriate		
0	0.123		190	0.00032	0.00017	
6	0.359	-	400	0.00078	0.00019	0
24	0.580	-	450	0.21	0.047	0
48	1.092	++++	840	260	31	125

	Challenger F control			Challenged N control	
Time in Hr.	Turbidity E 530	Pellicle	H.P.	Turbidity E 530	Pellicle
0	0.000			0.123	
6	0.037	-	0	0.334	-
24	0.544	++	50	0.744	R 1.
48	1.053	+++	125	0.852	-

F Challenger dose = 3,200 organisms.

With the origin of a fimbrial pellicle during the next 24 hr., successful outgrowth by the fimbriate cells was achieved, and at 48 hr. 31% of the total cells was fimbriate (see table 24). Thus, in the second-half of the growth period in mixed culture, successful outgrowth had occurred; even although the fimbriate cells were non-flagellate, they had managed to form a pellicle. The reason for this successful outgrowth by the fimbriate, non-flagellate strain may have been that the challenged strain, as tested in this experiment, was unable to form a flagellar pellicle. The control tubes for the flagellate, non-fimbriate strain showed only a slight rim formation at 24 hr., and neither a rim nor a pellicle at 48 hr. Control tubes of the fimbriate challenger at these times showed a ++ pellicle, which increased to a maximum ++++ at 48 hr.

Experiment 12.

In this experiment, the roles of the challenger and the challenged organisms in the previous experiment were interchanged. The two strains used, therefore, were Sa 635 fim-fla+rha+ (Sa 635/3) as L challenger, and Sa 635 fim+fla-rha- (Sa 635/5) as N challenged organism. At the time of challenge, the viable count of the N challenged cell was 235 x 10^6 cells per ml., and the challenger L dose was 2,500 flagellate organisms. Nutrient broth (A) was used and the only test condition investigated was aerobic static broth. Controls of the challenger flagellate strain alone showed that at both 24 and 48 hr., it formed an

Table 25. Competitive growth in mixed culture in aerobic, static broth between flagellate, non-fimbriate and fimbriate, non-flagellate strains of Salmonella typhimurium.

L = Sa 635 fla+ fim- rha+ v. N = Sa 635 fla- fim+ rha-

Time in hr.	Turbidity E 530	Pellicle	Counts x 10^6/ml.		% flagellate cells in population	H.P.
			Viable	Flagellate		
0	0.222		235	0.00025	0.00010	2-10
6	0.372	-	500	0.00046	0.00009	50
24	0.822	++	660	0.034	0.0032	125
48	1.215	"++++"	790	26	3.3	125

	Challenger L control.		Challenged N control.		
Time in hr.	Turbidity E 530	Pellicle	Turbidity E 530	Pellicle	H.P.
0	0.000		0.222	-	2-10
6	0.055	-	0.343	-	50
24	0.570	"+++"	0.716	+	125
48	0.591	"++++"	1.188	++++	250

L challenger dose = 2,500 organisms.

extensive pellicle of typical flagellar granularity. The challenged fimbriate strain controls showed that it formed a slight pellicle at 24 hr. which increased in size to a maximum at 48 hr. With these controls in mind, it is interesting to see that at 48 hr. successful competition by the flagellate cells had occurred, and that they formed 3.3% of the total population (table 25). Under these donditions, it was interesting to note that this outgrowth had apparently occurred by the ability of the challenger flagellate cells to form their heavy flagellar pellicle (an ability absent in this line in Experiment 11). Although the relative increase of the flagellate cells between 24 hr. and 48 hr. is significant, examination of table 25 shows that the fimbriate cells are also continuing to increase, and the mixed test broth haemagglutinating power has increased considerably. If this experiment had been of longer duration, it would have been interesting to see the struggle between these two strains.

In experiments 11 and 12, examination of rhamnose-fermenting and non-fermenting colonies by the "tile test" and "hanging-drop" test, for haemagglutination and motility respectively, showed that the single colonies were of the correct identity, being either fimbriate or flagellate but never flagellate and fimbriate.

SECTION III

The isolation and chemical structure of purified fimbriae.

At the outset of this investigation, the only reported purification method for fimbriae made use of electrophoresis at a high pH of II (Brinton, 1959). His findings indicated the presence of constituents other than protein. Lest this high pH might cause disaggregation of the constituents of this hypothetical complex it was decided to confirm or deny these findings by a purification method restricted to the pH region of neutrality.

Strains.

The two strains used were Escherichia coli 23 and Shigella flexneri FIaI each of which is strongly fimbriate, non-flagellate and non-capsulate. This precludes preliminary separation from at least some of the other surface components.

Inoculum.

The two strains were taken through serial 48 hr. broth subculture, until examination of single colonies plated on agar showed that the percentage of fimbriate cells was greater than 95%. Fully-fimbriate cultures were then maintained by serial 24 hr. subcultures in 100 ml. bottles of broth, checking the purity of the strain daily.

Growth media.

The nutrient broth (B) described in the methods section was used, incubating as described in the text. One ml. of the above cultures per Roux bottle was used as inoculum. Attempts to employ either solid media or artificially aerated cultures did not give greater yields

of fimbriae, although they increased the growth levels.

Defimbriation of fimbriate bacteria.

Assay of defimbriation.

Since fimbriae cannot be detected by a staining method, one is faced with the problem of assaying for successful defimbriation. The tile test, used to estimate the haemagglutinating activity of treated cells, initially gave misleading results. Reliance could not be placed on this test alone, because no matter how long the cells were treated, detectable levels of haemagglutinating activity persisted. Defimbriation techniques would be expected to snap off the fimbriae from the cell at their distal end. The residual stumps after such breakage would be subsequently more difficult to remove, and would account for this haemagglutinating activity. Parallel electron micrographs allowed one to estimate satisfactorily complete defimbriation as a standard reduction in haemagglutination titre as detected by the tile test.

Techniques.

The ideal method of defimbriation would be one which was speedy and which did not result in the breakdown of other cell surface components.

1. Heating.

Gillies and Duguid (1958) had shown that Shigella flexneri strains could be successfully defimbriated by heating at 90° for 30 minutes. This method, confirmed as successful, results in such gross contamination of the

fimbriae by denatured extracellular and intracellular materials that it is impossible to remove the adherent contaminating material even by repeated thorough washing with sodium acetate and distilled water.

2. Acid treatment.

This technique has been satisfactory for the removal of flagella (Stocker and Campbell, 1959) but did not work with fimbriae because of their acid tolerance.

3. Rubbing on stiff agar.

Stocker and Campbell (1959) found that repeated rubbing of a thick paste of flagellated cells on the surface of well-dried agar successfully detached flagella. This method proved suitable for the defimbriation of cells, but conditions could not be standardised easily. Also, since the cells did not produce many fimbriae on agar, this method was not pursued.

4. Mechanical methods.

The first investigated were those which might have been suspected as capable of defimbriating cells simply by their vigour. Attempted detachment of fimbriae by (a) high-speed centrifugation, (b) by continued shaking at 100 oscillations per minute on a reciprocating shaker and (c) repeated pipetting at high pressure through a fine orifice were all unprofitable, although the last-mentioned method resulted in a gradual slow defimbriation.

(d) The failure to detach fimbriae by constant vibration on a Mickle disintegrator was not surprising since the reciprocating shaker had not been successful.

The addition of ballotini beads to this system caused both detachment of fimbriae and cell breakdown. Suspension of the cells in I.5 M. sucrose during vibration on the Mickle proved a satisfactory procedure, no doubt because the increased viscosity of the sucrose solution resulted in greater shearing forces being generated in the shaken suspension. This method was not used because the subsequent isolation of the fimbriae from such a viscous solution proved difficult.

(e) Attempts to remove fimbriae by ultrasonic vibration on a Mullard machine operating at 20 Kilocycles per second were disappointing, loss of haemagglutinating activity being accompanied by cell wall breakdown. Such difficulty as was experienced in successful defimbriation no doubt reflected the relative difference in length of the two appendages. However, high-speed agitation of suspensions by Waring blendor has been a well-exploited method of detaching adsorbed bacteriophage (Hershey and Chase, 1952) fimbriae (Brinton et al, 1954) and flagella (Stocker and Campbell, 1959). Initial attempts to use this, assaying only by the tile test, had been disappointing, but reinvestigation using electron microscopical controls confirmed that fimbriae were completely detached after two minutes blending (Brinton et al., 1954).

Routine procedure.

Centrifuged, washed bacteria were resuspended in pre-chilled distilled water to a final volume of 60 ml.

This was blended in a 100 ml. vortex flask on a M.S.E. homogeniser at maximum speed (14,000 r.p.m. propeller speed) for two min.. The detached fimbriae were immediately separated from the intact cells by spinning in a refrigerated M.S.E. centrifuge at 5,200 G for 30 min.. The resultant supernatant contained fimbriae and other contaminating materials, mainly cell wall, as judged by electron microscopy.

Advantages of this routine.

The main advantage of this method is the short duration for completion of the shaving process. The bakelite container can also be surrounded by ice during blending, so that the temperature rise is negligible. Reduced temperature also increases the viscosity of a suspension and so increases the shearing forces. Chilling and suspension in distilled water reduces the activity of autolytic enzymes to a minimum. The misinterpretations which can occur in the absence of such simple precautions are shown in cell wall studies. The failure of other authors to find an R layer in Salmonella gallinarum was attributed to the activity of these enzymes (Weidel, Franck and Leutgeb, 1963). Although the fimbriae remained intact as judged by electron microscopy even in the absence of these precautions, they did prevent contamination of the fimbriae by cell wall breakdown products.

Analyses of crude fimbrial preparations.

Although treatment by blending caused no cell

death in the hands of other workers (Stocker and Campbell, 1959) it was apparent even from a visual examination of the slightly yellow supernatant after centrifugation at 5,200 G that cell breakdown had, in fact, occurred. This was further confirmed by the presence of cell wall material in preparations checked by electron microscopy. The crude preparations so obtained were centrifuged at speeds up to 15,000 G to remove large cell wall contaminants, and thoroughly dialysed against repeated changes of distilled water. The resultant suspensions, often apparently homogeneous when viewed by electron microscope, were analysed on several occasions. The results confirmed Brinton's finding of a low nitrogen content (1959). Estimation of the total nitrogen-dry weight content revealed consistently low values from 5.5-8.4%, with a mean of 7%, and carbohydrate -dry weight content (measured as glucose) from 3-21%, with a mean of 11%.

Treatment of crude fimbrial preparations with sodium dodecyl sulphate, lipase and ether.

The turbidity of such preparations was estimated and the percentage decrease in optical density calculated from comparison of control and test preparations under identical conditions. Equal volumes of sodium dodecyl sulphate (0.6%w/v), 0.02 M phosphate buffer, pH 6.0, and fimbrial suspension were incubated together at 37°. The turbidities of test and control suspensions, taken at 15 min. intervals up to 2 hr., showed that the final percentage turbidity decrease was always, approximately, 33%.

A similar experiment using the enzyme lipase (Light and Co.), 0.1% (w/v) in 0.02 M phosphate buffer, pH 7.5, gave a percentage decrease of 42% in the same time. Likewise, repeated ether extraction, before and after hydrolysis, removed a total of 42% of the dry weight. All these results were indicative of the presence of protein-polysaccharide-lipid complexes. Whether this was due to contaminating material or to the fimbriae themselves it was difficult to decide because our only criterion of purity, i.e. electron microscopy, was unreliable on its own.

Treatment of crude fimbrial preparations with amylase, pepsin and sodium dodecyl sulphate.

At this stage, since the analyses of crude fimbrial preparations had shown almost typical cell wall analyses, it was decided to treat such preparations by a method known to break down cell wall. A crude fimbrial preparation of E.coli 23 was concentrated by sedimentation at 105,000 G for 60 min., and the sediment resuspended in phosphate buffer, 0.2 M, pH 6.9, containing amylase at a concentration of 40 µg. per ml. This was then incubated overnight for 15 hr. at 37°, and then sedimented by centrifugation. The next stage was to incubate this sediment, resuspended in glycine-HCl buffer at pH2.0 for 15 hr. at 37°. The buffer contained 500 µg. of pepsin per ml. After digestion with amylase and pepsin, the preparation was divided into two, and a direct preparation for electron

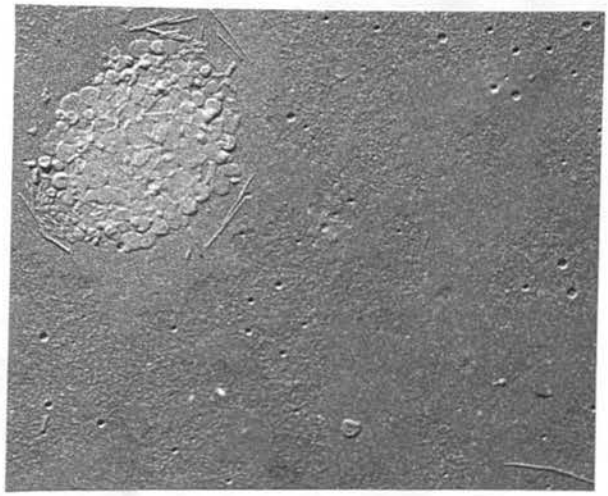


Fig.3a. Crude fimbrial preparation from E.coli 23, after treatment with amylase for 15hr. at 37°, followed by pepsin for 15hr. at 37°. This shows partially disaggregated cell wall material, and morphologically unchanged fimbriae.

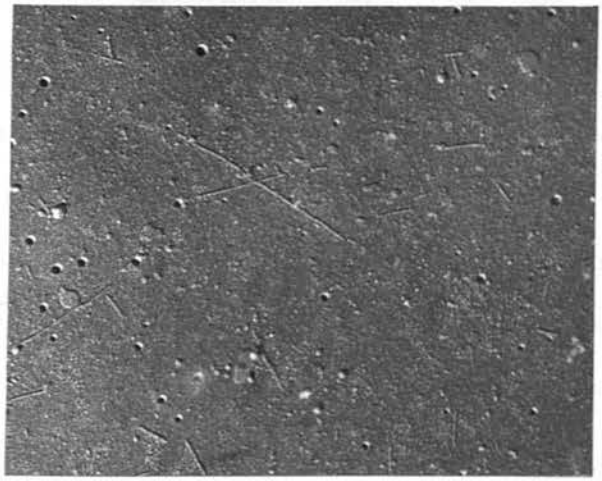


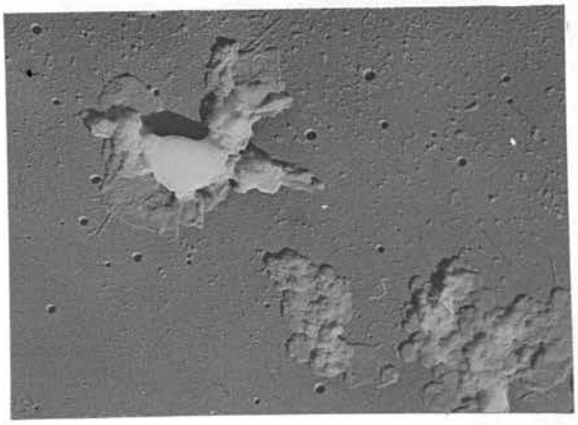
Fig.3b. As in (a), followed by treatment with 4% sodium dodecyl sulphate. The cell wall has completely disaggregated, but the fimbriae remain unaffected. Direct preparations made by agar filtration method.,(mag. x 24,000).

microscopical examination made of one by the agar filtration method. The other was then treated with 4% sodium dodecyl sulphate for 15 min. and a direct preparation made of this also. This method has been carefully standardised in cell wall studies (Weidel, Frank and Leutgeb, 1963) and so the contaminating cell wall material provided an excellent control. The figure 3a shows some degradation of the cell wall after treatment with amylase and pepsin but, although deformed, its structure is still quite clear. On the addition of sodium dodecyl sulphate, these structures disaggregate completely and the preparation shows no sign of cell wall structure at all. The fimbriae, clustered round the cell wall in figure 3a, are, possibly, thinner in diameter than control preparations, and this is true also of the fimbriae in the final preparation, fig. 3 b . While this might be an artefact resulting from the shadowing technique, it seems more likely that this is a direct effect of the chemical treatment. Most likely, it results from prolonged exposure to a low pH of 2.0.

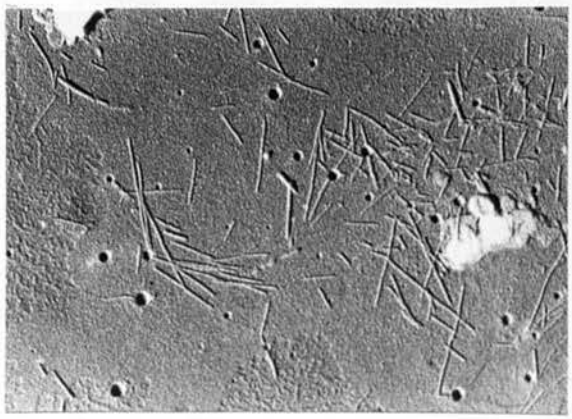
Purification of crude fimbrial preparations by differential centrifugation.

It was natural to attempt further purification by differential centrifugation since Weibull (1949) had successfully purified flagella in this way. After centrifugation at 15,000 G the fimbriae were concentrated to a volume of 100 ml. by dialysis against polyethylene glycol 6,000 (Kohn, 1959). After centrifugation at 15,000 G for 30 min. the supernatant was carefully removed and spun at

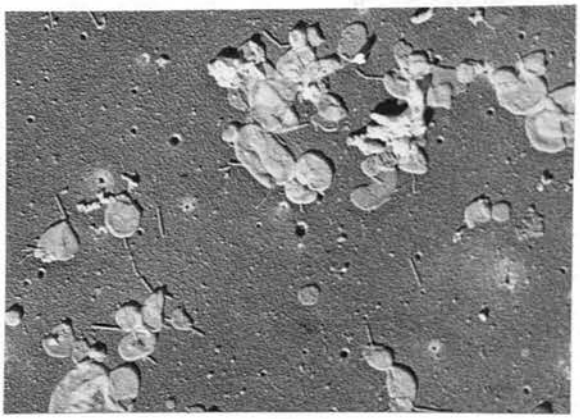
(a)



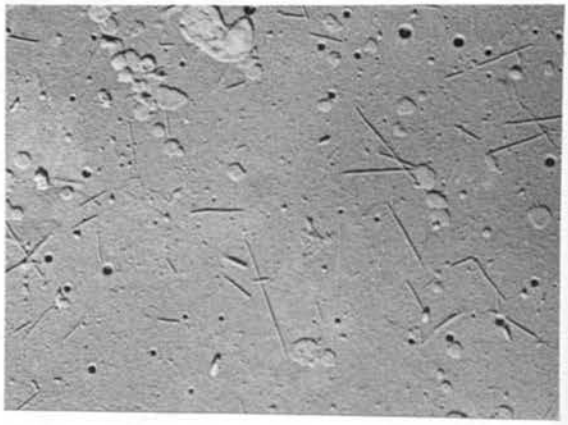
(b)



(c)



(d)



(e)

(f)

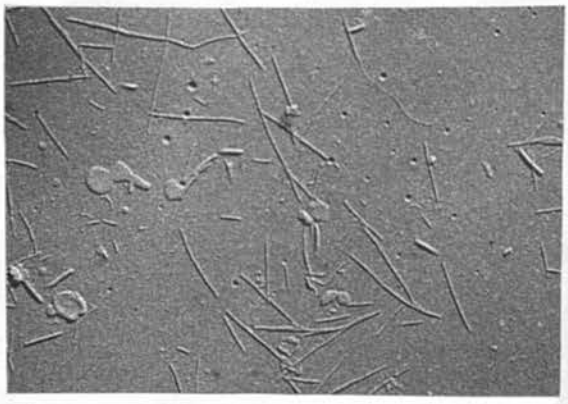


Fig.4. Differential centrifugation of fimbriae from E.coli AI22.
(a) sediment after centrifugation at 5,200 G x 30 min.
(b) supernatant " " " " " " "
(c) sediment " " " "15,000 G x 30 min.
(d) supernatant " " " " " " "
(e) sediment " " " "59,000 G x 30 min.
(f) supernatant " " " " " " "

59,000 G for 30 min. on a Spinco centrifuge model L. The sediments at 15,000 and 59,000 G and the supernatant after 59,000 G were prepared for electron microscopy. The photographs at these stages are shown in the accompanying figure 4 , showing that considerable purification is afforded by this scheme of differential centrifugation. All fimbrial preparations, therefore, were hereafter partially purified by retaining only the supernatant after 59,000 G. Further centrifugation on the Spinco at 105,000 G for times up to 120 min. did not quantitatively sediment the fimbriae from a suspension.

Preparations purified by differential centrifugation and analysed by the standard methods previously outlined, showed a total nitrogen-dry weight content of 12% and always less than a 1% carbohydrate-dry weight content.

Treatment of partially purified preparations with detergent.

Fimbriae from E.coli 23 were partially purified by the above plan and sedimented by centrifugation at 105,000 G for 60 min. The sediment, resuspended in distilled water, was split into two parts, one of which was used as control preparation. To the other an equal volume of 4% sodium dodecyl sulphate in water was added. The two preparations were incubated at 37° for 60 min., when a direct preparation of each was made for electron microscopy. The figures 5a and 5b show that anatomically, as judged by electron microscopy, this treatment had no effect on the fimbriae which appear of normal thickness.

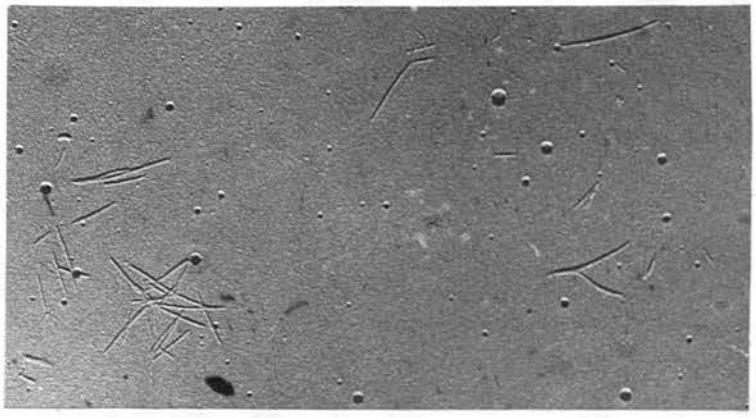


Fig.5a. Fimbriae from E.coli 23, partially purified by differential centrifugation, sedimented at 105,000 G., and resuspended in Na-K phosphate buffer, pH 7.5.

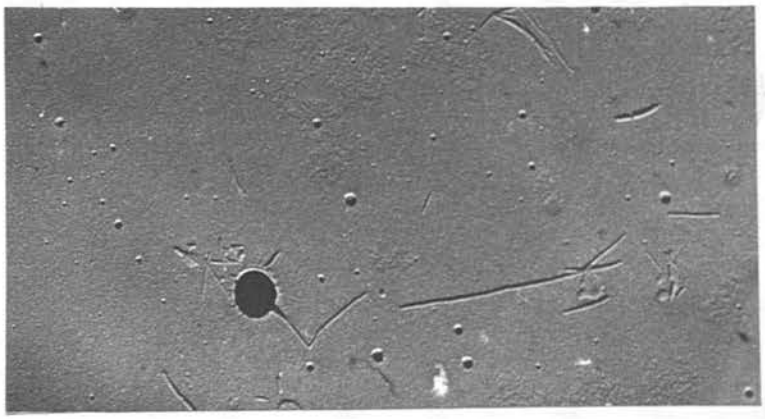


Fig.5b. As in (a), after treatment with 2% sodium dodecyl sulphate for 1 hr. at 37°. Direct preparations made by agar filtration method. (mag. x 24,000).

The finding of a total nitrogen-dry weight content of 12% in fimbrial preparations partially purified by differential centrifugation indicated a protein content of 75%. The reduction in turbidity after treatment of crude fimbrial preparations with detergent, lipase and ether, suggested that these were contaminated with 30-50% of cell wall or other lipoprotein material. This was confirmed by the electron microscopical evidence that fimbriae were morphologically unaltered by sodium dodecyl sulphate. Although the preparations were by now considerably purified, it was apparent that differential centrifugation had been only partially successful. The distorting effects caused by non-particulate material, not seen by electron microscope, and contaminating particles sedimenting at the same rate as the fimbriae, made it necessary to remove them.

Possible approaches to further purification.

Particulate cell antigens have been more commonly purified by differential centrifugation, isoelectric precipitation, electrophoresis and ultracentrifugation. The requirement for a purification method in the region of neutrality excluded some of these techniques. A communication was made claiming the isolation of fimbriae in pure state by isoelectric precipitation at pH 4.0 (Brinton and Stone, 1961). Analyses indicated a protein content of 98%.

I. Counter-current distribution.

This has been successfully used for the separation of particulate components of large molecular weight in a mixture, using immiscible polymers such as dextran and methylcellulose or polyethylene glycol. In this way, viruses and brain microsomes have been purified, and even strains of bacteria with different surface determinants, (Albertsson, Hanzon and Toschi, 1959, Albertsson and Frick, 1960) and Albertsson and Baird, 1962). This latter work reports that a single strain of E.coli K12 58 Hfr separated into two distinct peaks, whereas E.coli K12 C600 gave a single peak corresponding to the second peak of K12 58 Hfr. E.coli K12 C600 is a stably fimbriate strain and if E.coli K12 58 Hfr is a typical phase-varying fimbriate strain, then this differential behaviour might express their different surface configurations and allow a classification of the two kinds of fimbriate cell, E.coli fim^{0}+ and fim+. The application of these phase systems, using high molecular weight polymers, to the separation of particulate components is neglected because it is somewhat difficult to isolate the particular component from its suspending organic phase.

The advantage of a two phase system in which only one of the phases is organic is obvious, and such systems have been effectively applied to the purification of viruses from contaminating non-viral material using water and fluorocarbons. The two phases were homogenised on a blendor

and, on separation, non-viral material was retained in the organic phase and the virus concentrated in the aqueous phase (Gessler, Bender and Parkinson, 1956). Indications of a high fimbrial protein content suggested that this method might provide a satisfactory purification scheme.

Arcton 113 is a fluorocarbon, specific gravity of 1.4, (CF_2Cl-CCl_2F), manufactured by Imperial Chemical Industries, England. A crude fimbrial preparation (after centrifugation at 5,200 G) was added to 5 ml. of arcton and the two blended by a M.S.E. homogeniser for 30 seconds at maximum speed. The aqueous upper layer was separated by centrifugation at 2000 G for two min., and the process repeated with fresh arcton four times in all. The turbidity of the aqueous fimbrial phase during the procedure fell by 60-90%, depending on the crudity of the preparation. Electron microscopy of the two phases showed that the water phase contained mostly fimbriae, while the lower arcton phase contained large amounts of cell wall debris. The decrease in optical density was accompanied by a slight increase in the total nitrogen-dry weight content (6.5-10%), and the haemagglutination titre remained as before. The results with this method were somewhat irregular and it was not investigated farther, but its potential as a very useful method, if extensively examined and standardised, seems likely.

II. Column chromatography.

Purification of particulate material - e.g. cell wall, flagella - by this means was not known. Its applicability in

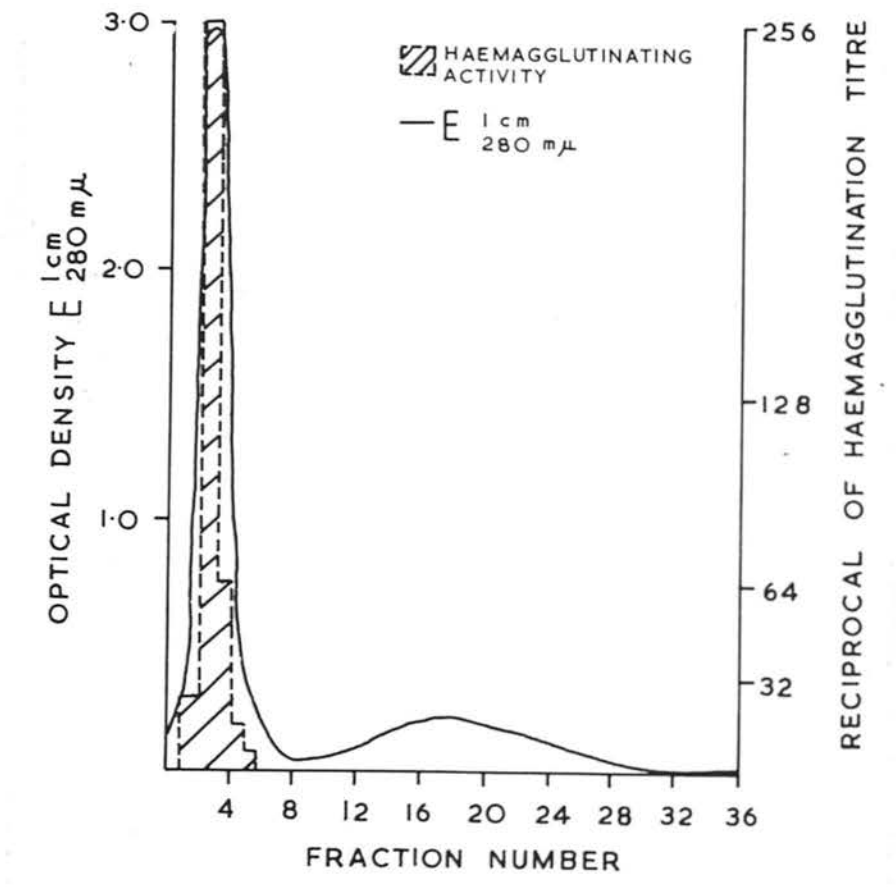


Fig.6. Fractionation of fimbrial preparation from 48 hr. culture of E.coli 23 on CM - cellulose, equilibrated at pH 6.0, with 0.02 M Na-K phosphate buffer. Elution with 0.02 M Na-K phosphate buffer, pH 6.0.

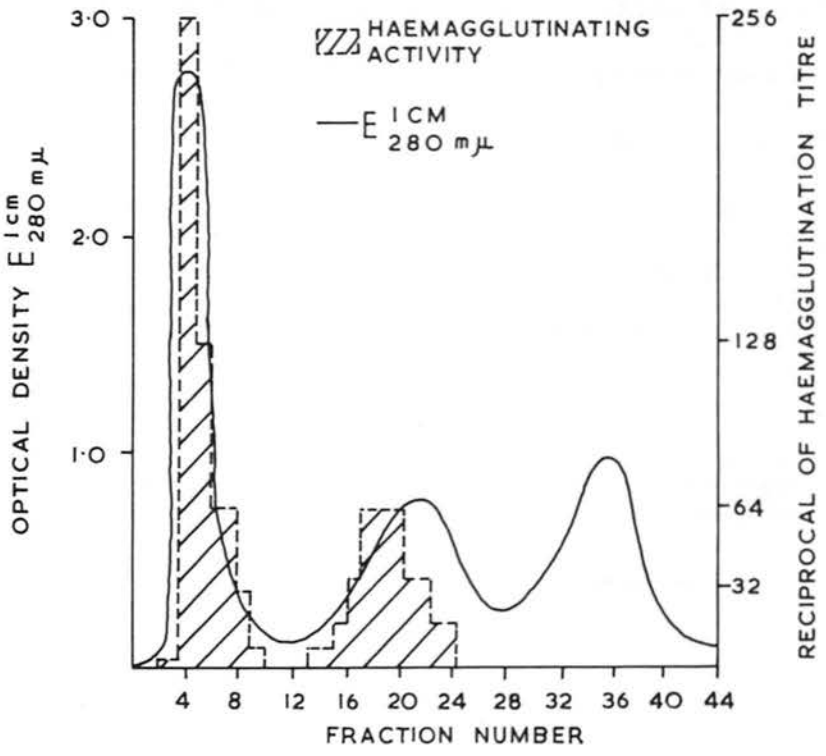


Fig.7. Fractionation of fimbrial preparation from 48 hr. culture of E.coli 23 on DEAE - cellulose, equilibrated at pH 7.0 with 0.02 M Na-K phosphate buffer. Elution with salt gradient, as described in text.

the isolation of homogeneous fimbrial preparations was investigated.

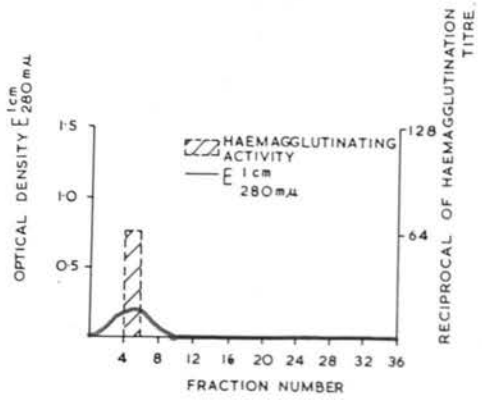
a. Fractionation of fimbriae from E.coli 23 on carboxy-methyl (CM)-cellulose.

Fimbrial preparations from 48 hr. cultures, partially purified by centrifugation at 59,000 G, were fractionated. The figure 6. shows the distribution pattern, of the fractions read at 260 and 280 mμ, when fimbrial preparation was applied to a CM-cellulose column and eluted with 0.02M Na-K phosphate buffer, pH6.0. Almost all of the material came through with the starting buffer and was not retarded by the column. The leading boundary showed haemagglutinating activity which was absent in the second peak. The material which came through in the first peak had not been purified by this single passage as shown later by agar gel diffusion tests.

b. Fractionation of fimbriae from E.coli 23 on diethylaminoethyl (DEAE)-cellulose.

A similar E.coli 23 preparation was applied to an equilibrated, DEAE-cellulose column, 1 x 12 cm., and adsorption of fimbriae judged to have occurred at pH7.0 by the absence of haemagglutinating activity in the eluate. Attempts to elute this by pH gradient resulted in a slight haemagglutinating activity over many fractions. Application of a salt gradient, as described in the text, eluted a major and a minor peak, each of which was haemagglutinating, followed by a third peak which was always non-haemagglutinating, (see figure 7). The size of this third peak varied

(a)



(b)

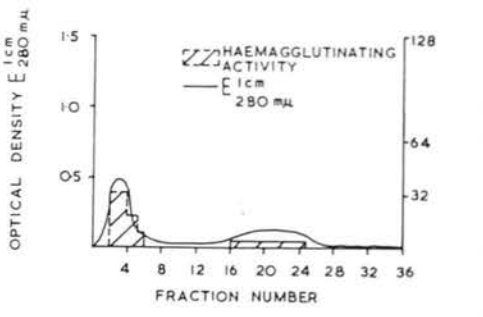


Fig.8. Pooled fractions of haemagglutinating peaks of E.coli 23 fimbriae, after fractionation on DEAE-cellulose, rechromatographed on DEAE-cellulose under identical conditions as in Fig.7.
(a) - peak i.
(b) - peak ii.

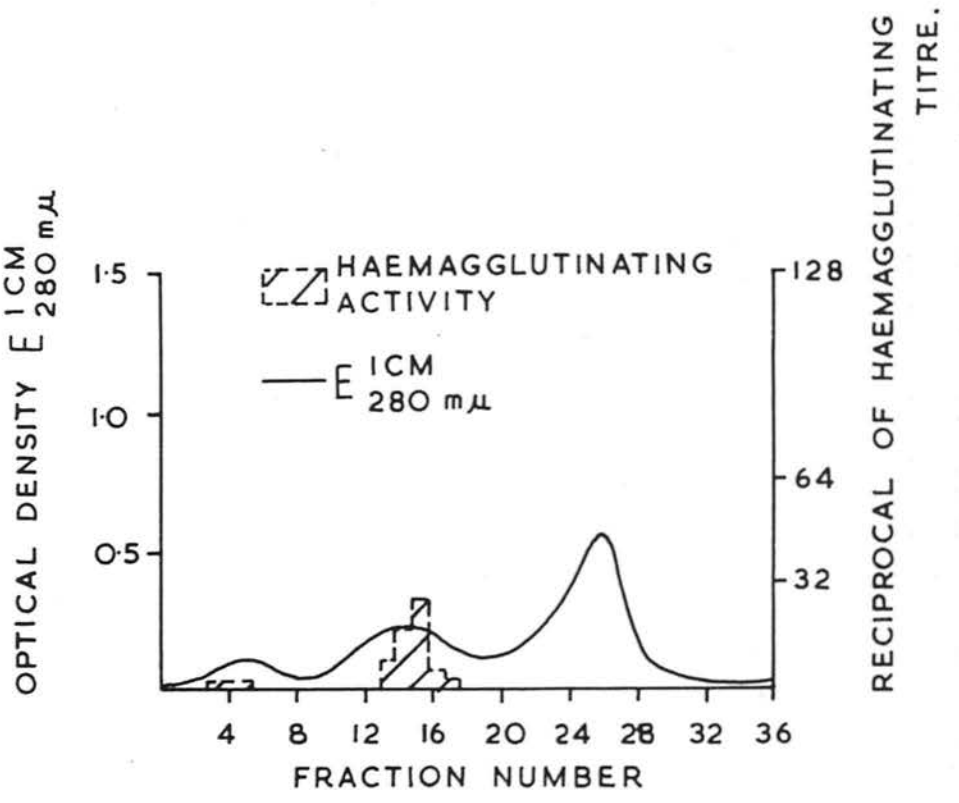


Fig.9. Fractionation of fimbrial preparation from 48 hr. culture of Shigella flexneri Flal on DEAE-cellulose, equilibrated at pH 7.0 with 0.02 M Na-K phosphate buffer. Elution with salt gradient, as described in text.

according to the degree of contamination with nucleic acid. The eluate volumes for these two haemagglutinating peaks were II-42ml. and 75-115ml., corresponding to molarities of 0.11 and 0.37M and 0.88M NaCl for peaks I and II respectively.

The fractions of each of the two peaks were pooled, concentrated by dialysis against polyethylene glycol to volumes of 5ml. and re-equilibrated against 0.02M Na-K phosphate buffer at pH7.0, so that they could be re-chromatographed under identical conditions. The results are shown in figures 8a and 8b. The major peak reaches approximately the same final positions as in figure 7 although the amount of protein recovered and the haemagglutinating activity detected have both fallen during this refractionation process. The second peak gives the same pattern as if a crude preparation had been chromatographed, e.g. two peaks. It seems that the homogeneity of the major peak has been proved by its monophasic behaviour during rechromatography.

Fractionation of fimbriae from Shigella flexneri FIaI on DEAE-cellulose.

Fimbrial preparations from partially purified 48 hr. cultures of Shigella were applied to equilibrated DEAE-cellulose column and the fractions analysed. These showed a different behaviour from E.coli fimbriae. On application of a salt gradient, a minor peak, which once showed weak haemagglutination, and a major secondary peak of stronger haemagglutinating activity were eluted. A third non-

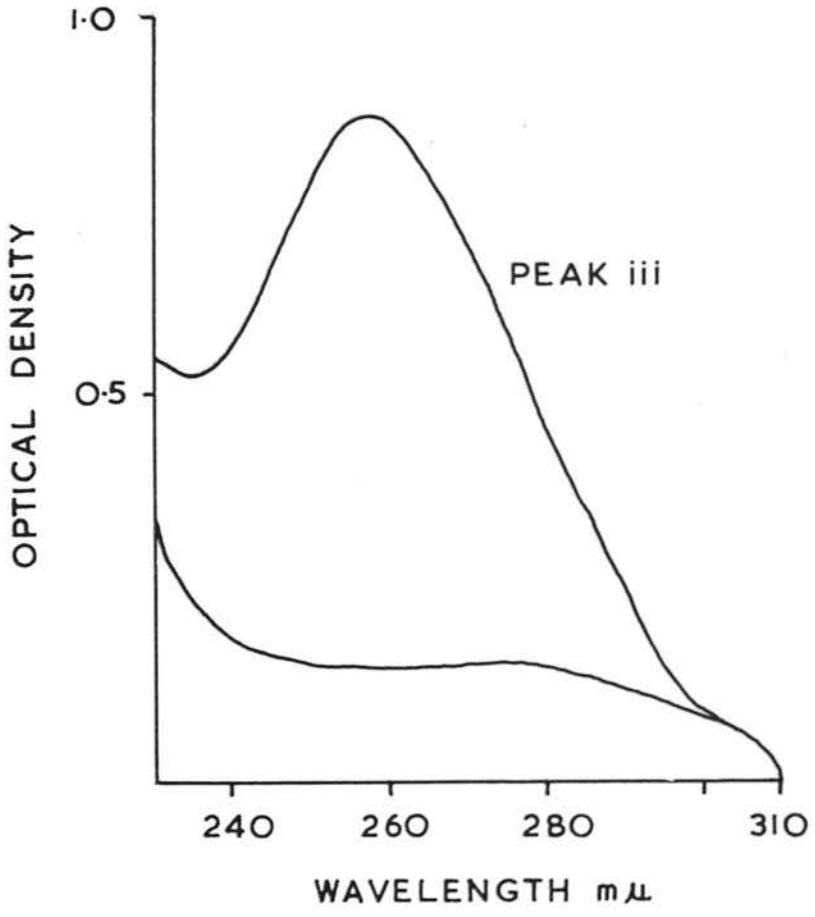


Fig.10. Absorption spectra of crude fimbrial preparation from E.coli 23, and non-haemagglutinating peak iii obtained after fractionation on DEAE-cellulose and eluted by salt gradient.

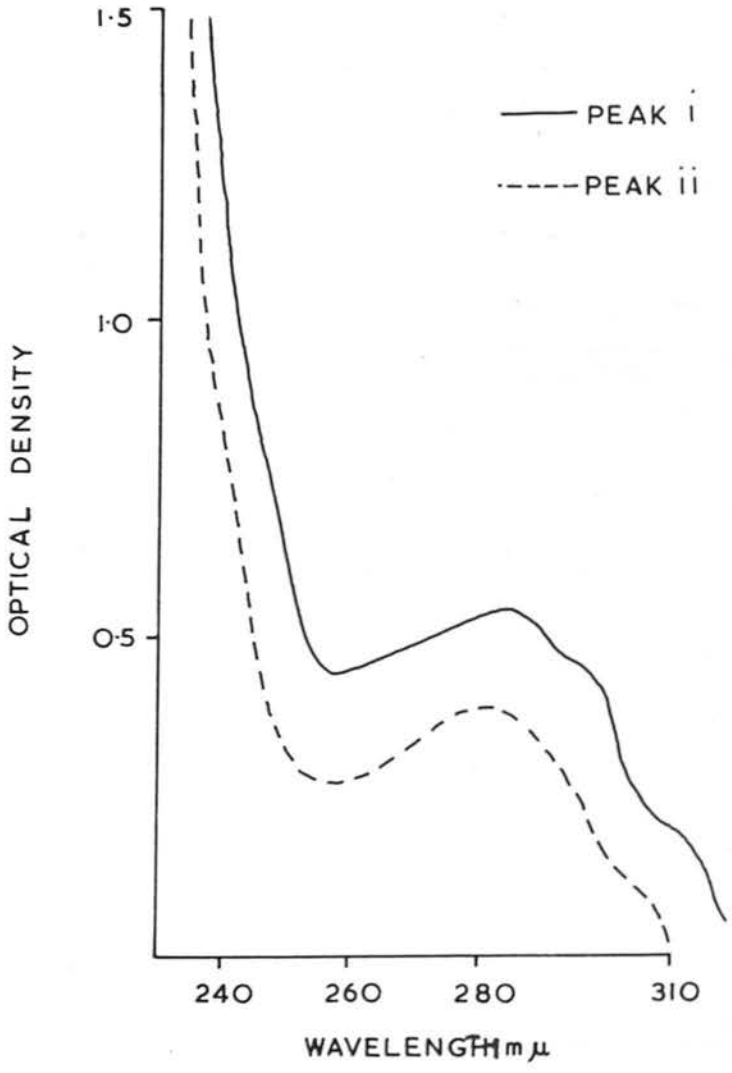


Fig.11. Absorption spectra of haemagglutinating peaks i and ii from E.coli 23, obtained after fractionation on DEAE-cellulose and eluted by salt gradient.

haemagglutinating trailing peak was also found. The pattern is shown in figure 9.

Absorption spectra of the three peaks obtained after fractionation on DEAE-cellulose.

Three such peaks as were obtained by this fractionation procedure from E.coli fimbriae were diluted in distilled water, so that the extinction values at 260 and 280 mμ on a spectrophotometer were similar for all three. Continuous spectra were plotted from readings at intervals of 1mμ wavelength from 250-280 mμ and at 5mμ intervals on either side of these two limits from 230-310 mμ. When a crude preparation of approximate nitrogen-dry weight content 6-8% was taken its absorption spectrum fell gradually to 250 mμ, then levelled out over the region 250-270 mμ and declined rapidly after 280 mμ. This was probably due to the masking effect of the major contaminants, cell wall, cytoplasmic membrane and nucleic acid. (see figs.10 and 11).

The absorption spectra of the two haemagglutinating peaks gave absorption maxima at 278 mμ, i.e. in the region of characteristic protein maxima. The third, non-haemagglutinating peak gave maximum absorption readings at 260 mμ, characteristic of mucleic acid material. This third peak, when tested by agar gel diffusion, gave no band of precipitation (see later section), and when viewed by electron microscope revealed no particulate material.

Analysis of fimbrial preparation purified by column chromatography.

A purified fimbrial preparation of E.coli 23 was

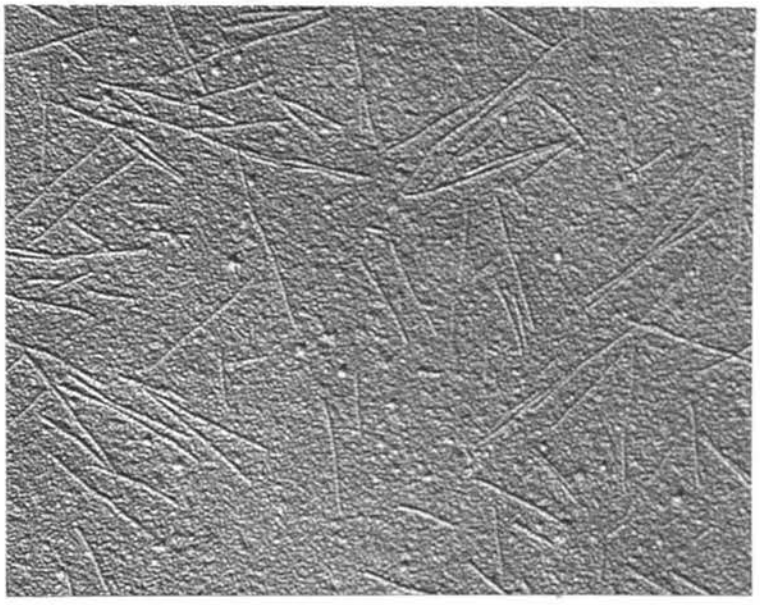


Fig.12. Fimbriae from E.coli 23 after differential centrifugation, chromatography on DEAE-cellulose and dialysis against distilled water (mag. x 48,000).

analysed after dialysis against distilled water. Such preparations gave a total nitrogen-dry weight content of 14.8-15.2%, which was indicative of 95% protein. Negligible amounts of carbohydrate and phosphorus were detected.(fig.12).

Detection of amino acids in fimbriae.

Purified fimbriae, after hydrolysis with 6N HCl for 24 hr., were assayed for amino acids by two-dimensional chromatography. The qualitative analyses revealed the following amino acids present in large amounts (as detected by size and intensity of spots on staining with ninhydrin): aspartic acid, glutamic acid, serine, glycine, alanine, threonine and valine. Spots staining less definitely were found at positions corresponding to the amino acids arginine, lysine, tyrosine and histidine. In the region of the amino acids leucine, isoleucine and phenylalanine, definite coloration was observed strong enough to suggest that amino acids were present. This may have been the leucines or phenylalanine, or all three. The strong absorption at 280 mμ of purified peaks from DEAE-cellulose fractionation suggested that the aromatic amino acids were present. Tyrosine is present in small amount, and, perhaps, phenylalanine. Tryptophane is not detected by these means.

Agar-gel diffusion tests as a criterion of purity.

Although these fimbrial preparations were agglutinated in the presence of antifimbrial serum in tube tests, such a method is limited because it gives no indication of the

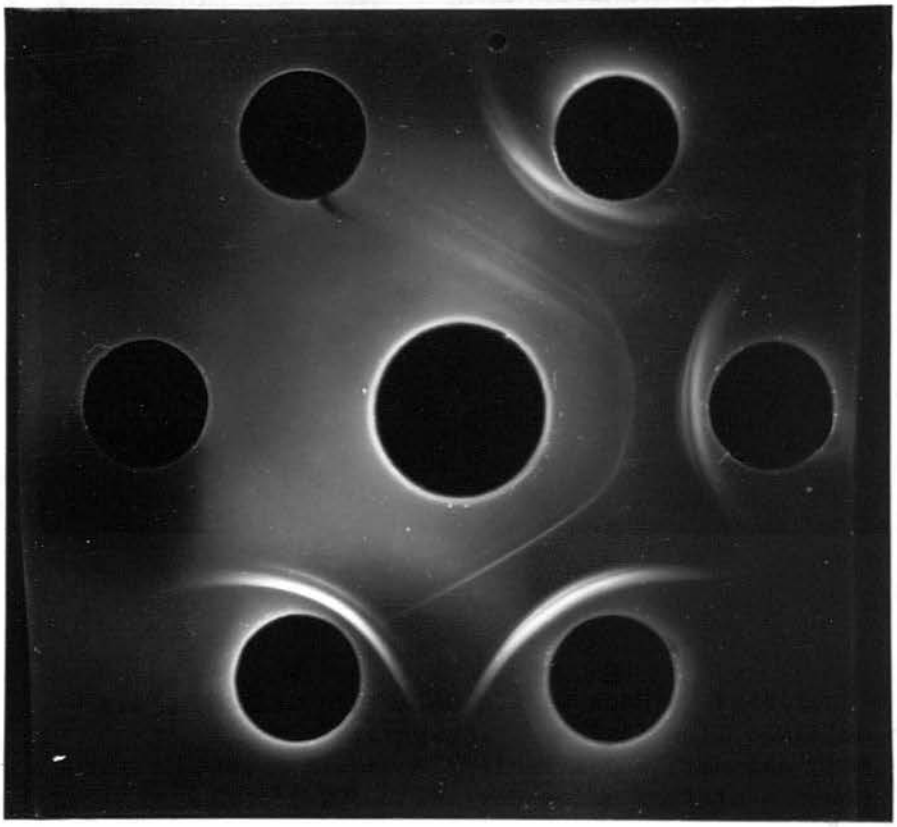


Fig.13. Double diffusion plates showing reactions of fimbrial antigens. Central well contains antiserum prepared against crude fimbriae from E.coli 23. Antigen wells contain a crude fimbrial preparation from E.coli 23 which is in (1) untreated, (2) treated with 0.005 N HCl. at 37° for 5 min, (3) heated at 60° for 3 hr., (4) heated at 100° for 3 hr., (5) autoclaved at 120° for 30 min. and (6) control containing phosphate buffer.
N.B. Antigen wells are numbered in a clockwise direction.

number of antigens present in a preparation. To overcome this limitation, use was made of the agar-gel diffusion technique which requires only small amounts of antigen and antiserum (Ouchterlony, 1948).

Initially, it seemed that particulate fimbriae might not be suitable for gel-diffusion work, but this was not so. The antisera used were high-titre sera prepared against (a) a crude fimbrial preparation of E.coli 23 after partial purification by differential centrifugation (b) an absorbed fimbrial serum prepared by the method of Gillies and Duguid (1958). Since E.coli 23 never became completely non-fimbriate on agar subculture, the fimbriate phase was sought by culturing in 1% glucose broth for 18 hr. on a reciprocating shaker at 100 oscillations per min., all three factors normally contributing to the selection of the non-fimbriate phase.

Proof that the main line of precipitation was fimbrial.

Since it is almost impossible to denature fimbriae by specific chemical agents or even prolonged heating, it was difficult to prove that the strongest band of precipitation in agar-gel diffusion tests (i.e. the line least removed from the antigen well) was caused by the interaction of fimbrial antigens and antiserum. A crude fimbrial preparation was treated with 0.005 N HCl at 37° for 5 min. to show that this routine method of destroying H antigen (Duncan, 1935) did not remove the haemagglutinating activity or destroy the antigenicity of fimbriae. The same preparation was heated at 60° and 100° for times up to 3 hr. and also autoclaved

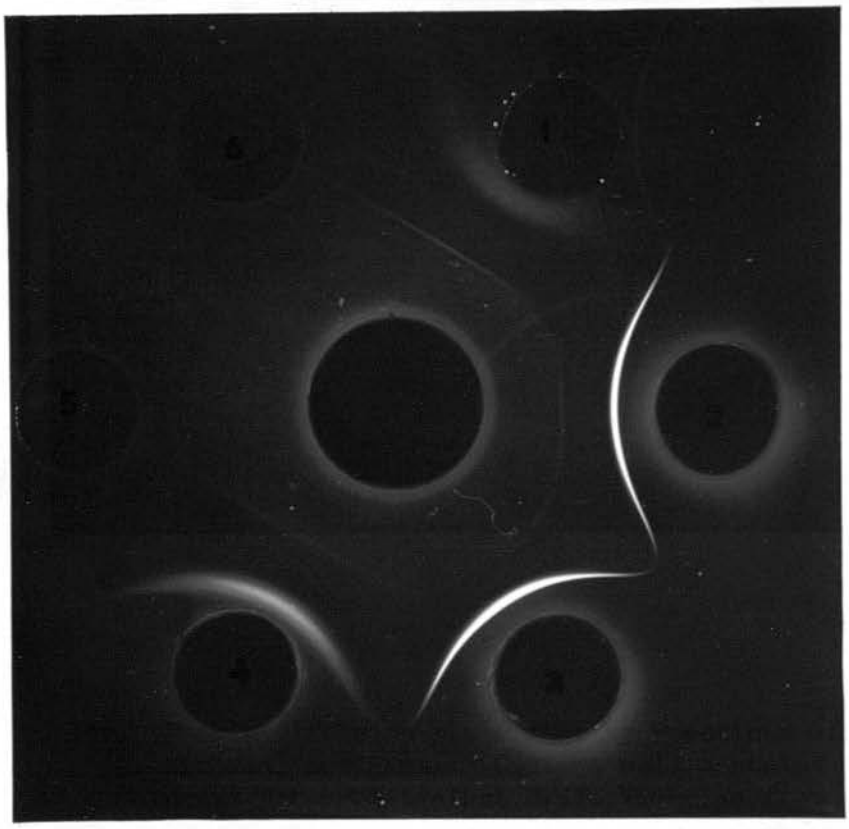


Fig. 14a. Double diffusion plate showing reactions of fimbrial antigens. Central well contains antiserum prepared against crude fimbriae from E.coli 23. Antigen wells contain (1) fimbrial preparation from E.coli 23, supernatant after centrifugation at 105,000 G x 30 min., (2) sediment after 105,000 G x 30 min., (3),(4) and (5) peaks i, ii and iii, respectively, concentrated after fractionation on DEAE-cellulose, and (6) control containing phosphate buffer.

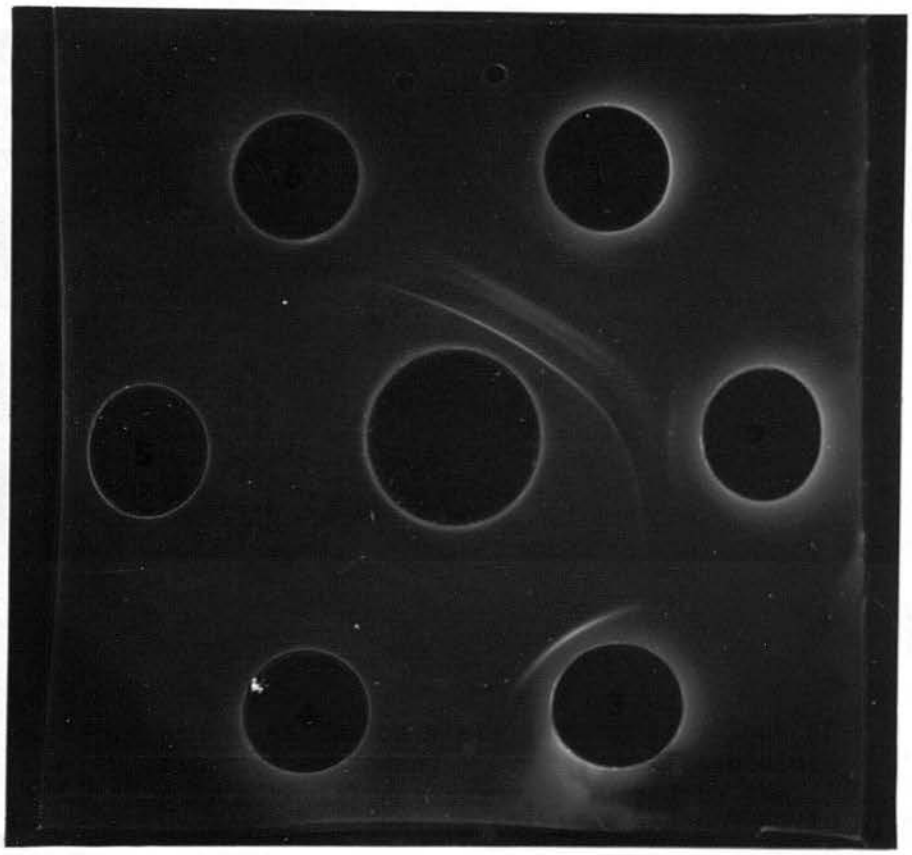


Fig. 14b. Double diffusion plate showing reactions of fimbrial antigens. Central well contains pure fimbrial antiserum, prepared by the absorption technique (Gillies and Duguid,1958). Antigen wells contain (1) fimbrial preparation from E.coli 23, supernatant after centrifugation at 105,000 G x 30 min., (2) sediment after 105,000 G x 30 min., (3),(4) and (5) peaks i,ii and iii, respectively, concentrated after fractionation on DEAE-cellulose, and (6) control containing phosphate buffer.

at 120° for 30 min. At the end of these times, the tests were plunged into ice-cold water to stop further action, and the haemagglutinating activity of all samples assayed. Only after heating at 100° for 2 - 3 hr. was a slight reduction noted in the H.A. titre of the preparation. These treated samples were then used as antigens against crude fimbrial antiserum. The antigens responsible for the main band of precipitation were not destroyed by any of these methods, except autoclaving - see fig.13. (However, Gillies and Duguid claimed that the antigenicity of Shigella fimbriae was destroyed after 2 hr. at 100°). The thermostability may be considered as evidence for fimbrial characterisation.

Fig.14a. shows the results obtained using an antiserum prepared against crude fimbriae. The four antigens - supernatant after centrifugation at 105,000 G x 30 min. the partially purified fimbriae sedimented at this speed, and peaks i and ii after fractionation on DEAE-cellulose - all produced a common band of precipitation showing that the supernatant after 105,000 G still contained significant amounts of fimbriae. The lines produced by this supernatant and also by peak ii were, however, somewhat diffuse, which possibly reflects a more dilute concentration of antigen. The success of differential centrifugation was a variable factor, but this particular preparation was clearly well-purified as evidenced by the presence of only one additional, weak line given by the first two antigens. This second line is also present in

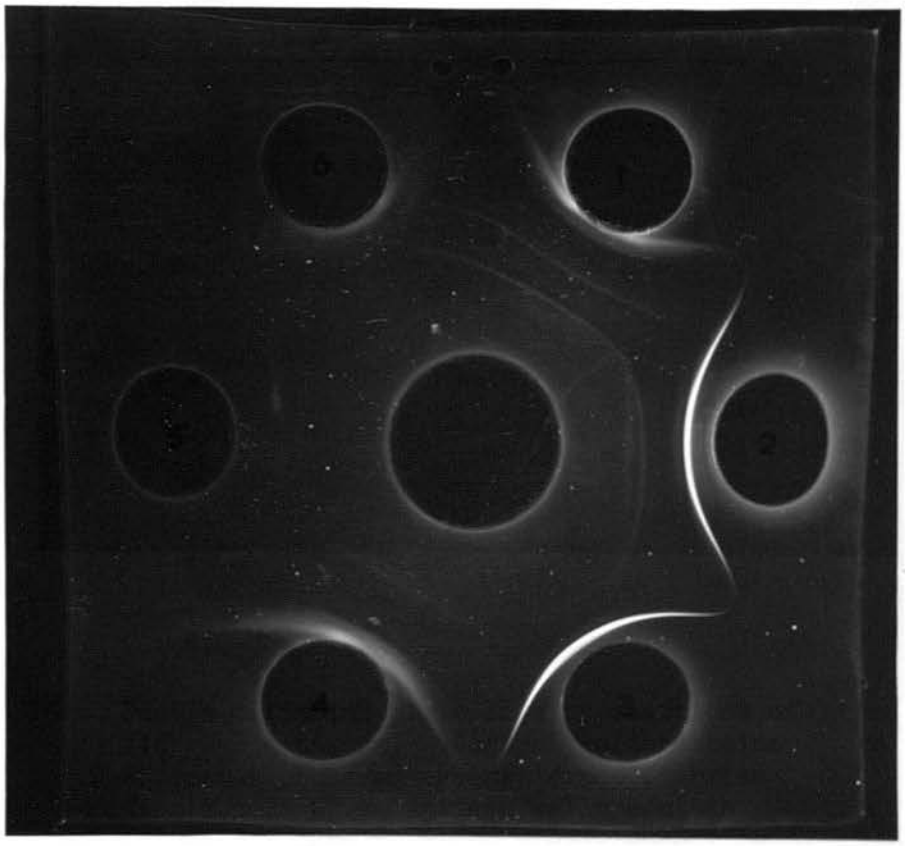


Fig. 15a. Double diffusion plate showing reactions of fimbrial antigens. Central well contains antiserum prepared against crude fimbriae from E.coli 23. Antigen wells contain (1) crude fimbrial preparation, supernatant after centrifugation at 59,000 G x 30 min., (2) sediment after centrifugation at 105,000 G x 30 min., (3) and (4) peaks i and ii, respectively, concentrated after fractionation on DEAE-cellulose, (5) crude fimbriae from S.typhimurium Sa 635 fim+ and (6) crude fimbriae from Shigella flexneri Fla1.

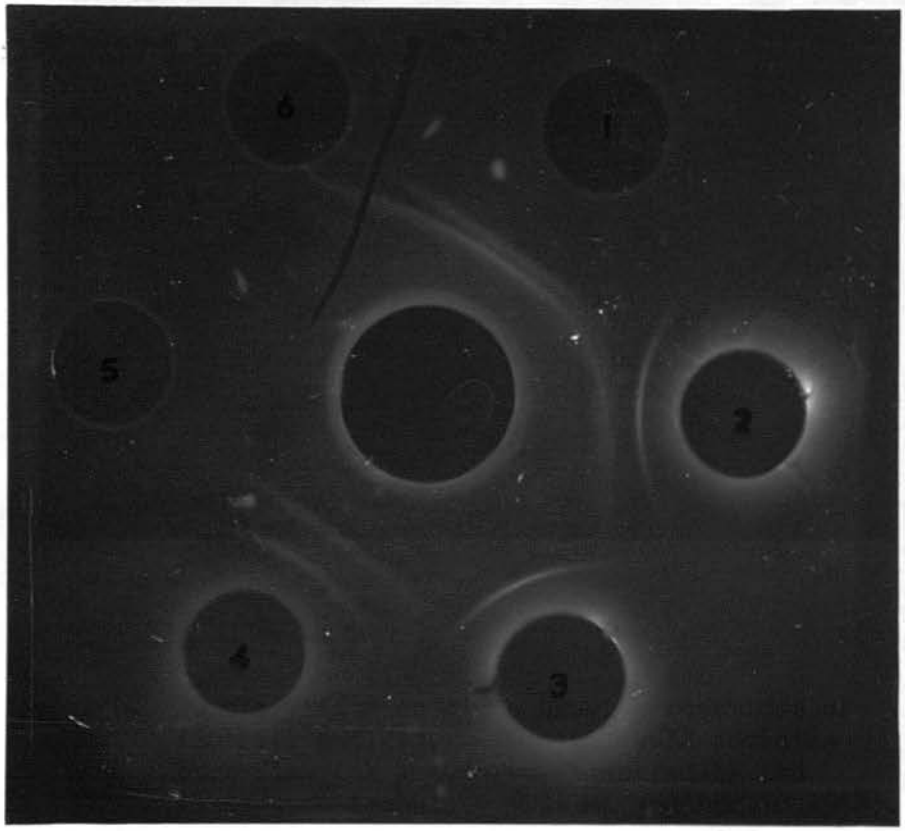


Fig. 15b. Double diffusion plate showing reactions of fimbrial antigens. Central well contains pure fimbrial antiserum, prepared by the absorption technique (Gillies and Duguid,1958). Antigen wells contain (1) crude fimbrial preparation, supernatant after centrifugation at 59,000 G x 30 min., (2) sediment after centrifugation at 105,000 x 30 min., (3) and (4) peaks i and ii, respectively, concentrated after fractionation on DEAE-cellulose, (5) crude fimbriae from S.typhimurium Sa 635 fim+ and (6) crude fimbriae from Shigella flexneri Fla1.

peak ii, but absent from peak i, which has behaved as a monovalent antigen. The non-haemagglutinating peak iii gave no lines of precipitation with this antiserum showing that its components were non-antigenic. Fig.14b. represents the same test antigens reacting against the absorbed fimbrial antiserum. The bands of precipitation, however, are much weaker, and this difference must reside, not in the antigens, but in the two antisera.

Figs. 15a. and 15b. compare the efficacies of the two antisera. In fig.15a, the crude fimbrial preparation (after centrifugation at 59,000 G x 30 min.) in well I induces, in addition to the main fimbrial band, at least four weaker bands. When centrifuged at 105,000 G x 30 min., one of the minor components disappears. Peaks i and ii, obtained by fractionation on DEAE-cellulose, behave as before. Crude Salmonella fimbriae in well 5 (i.e. the supernatant obtained after defimbriating the transductant S.typhimurium Sa 635 fim +) show no cross-reaction with this E.coli fimbrial serum. Shigella fimbriae (well 6) show a faint, diffuse band of precipitation slightly distant from the antigen reservoir, at the site of fimbrial interaction. Fig.15b. represents the behaviour of the same antigens with absorbed "fimbrial" antiserum. However, even the major bands are seen this time only with difficulty. These experiments suggest that it is not reliable to use a fimbrial antiserum obtained by absorption of the serum, prepared against a fimbriate organism, with the phenotypically non-fimbriate organism. Although only a small amount of haemagglutination

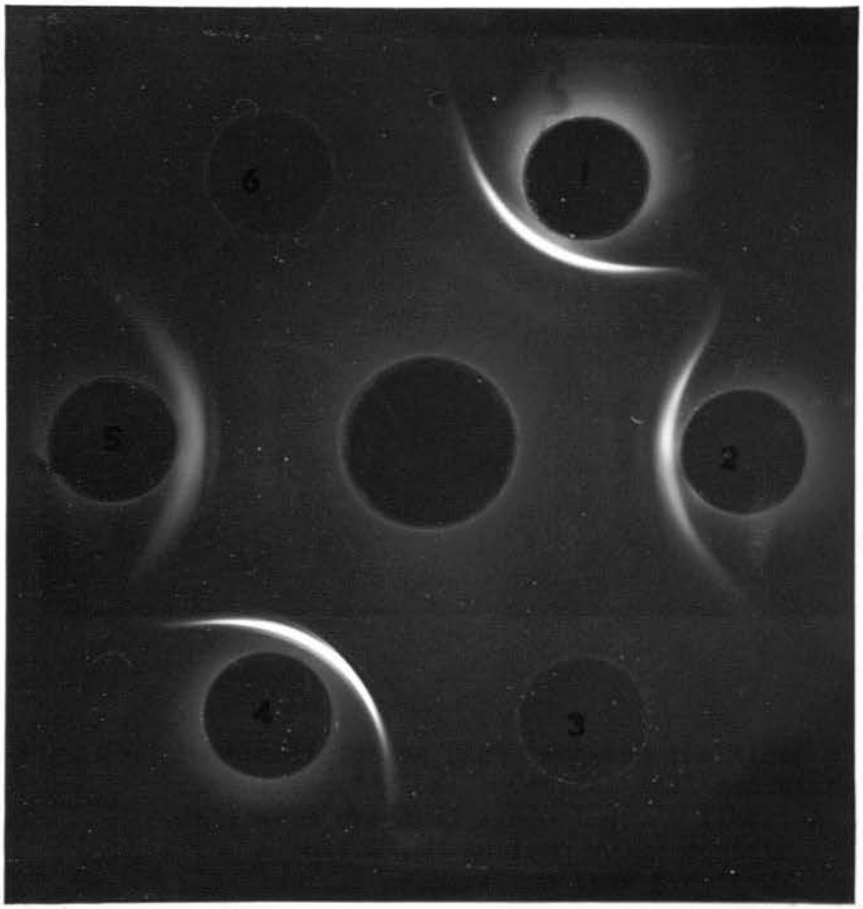


Fig. 16. Double diffusion plate showing reactions of fimbrial antigens. Central well contains antiserum prepared against crude fimbriae from E.coli 23. Antigen wells contain (1) fimbrial preparation from E.coli 23 partially purified by differential centrifugation, (2) as in (1) after chromatography on CM-cellulose, (3) as in (1) after chromatography on CM-cellulose, concentrated and rechromatographed on DEAE-cellulose, (4) and (5) peaks i and ii, respectively, concentrated after fractionation on DEAE-cellulose, and (6) control containing phosphate buffer.

was detected after E.coli 23 had been grown in shaken, glucose broth for 18 hr. (using an inoculum after several agar subcultures) a considerable amount of fimbrial antibody has been removed during absorption. This removal of fimbrial antibody results in a less intense band than when a crude fimbrial antiserum was used, and is, therefore, indirect proof of the identity of the fimbrial band.

Fig.16. shows the results of attempted removal of the minor antigenic components in the fimbrial preparations by primary fractionation on CM-cellulose. Antigen well I contains the partially purified fimbrial preparation, antigen well 2 the same preparation after CM-cellulose fractionation. This latter fraction shows, as well as the main fimbrial antigen band of precipitation, two minor components so that fractionation through this cellulose exchanger has not purified the fimbriae to a monovalent state. When passed through DEAE-cellulose, this fraction lost both its haemagglutinating activity and its antigenicity, as shown from well 3. Peaks i and ii, included as controls, show their typical behaviour, although an additional minor component may be present. Serial fractionation through the two cellulose exchangers took in all 7 - 8 days for completion and it is not surprising that the recovered material, tested in reservoir 3, lacked activity.

DISCUSSION

It has been possible to transduce fimbriation to the majority of the permanently non-fimbriate S.typhimurium strains. This ability to be transduced was not correlated with any character common among the recipient strains, such as phage-type, fermentation-type, animal source or age of isolate. With the exception of motility, which will be discussed later, it seems that the only requirement is for the recipient strain to possess the correct surface for the adsorption of the transducing phage, i.e. the possession of somatic antigen XII_2 (Lederberg and Edwards, 1953). That this process is transduction, and not some of the other methods of genetic transfer discussed in the introduction, emerges from the following points. Since the origin of fimbriate cells is a low-frequency event (one transduction per $5 \times 10^7 - 10^8$ infected cells), this cannot be phage-conversion in which every particle is active in exerting its effect. Attempts to transfer fimbriation to the recipient FIRN strains at low multiplicity were unsuccessful. Another alternative mechanism that can be excluded is high-frequency restricted transduction, because this rarely results in the integration of the transduced fragment into the recipient strain. The stability of the fim gene in a number of fimbriate transductants was examined by subjecting them to a series of rapid subcultures through agar and broth. Single colonies randomly selected and tested after such procedure were all fimbriate, suggesting that the fim gene had been integrated in the bacterial

chromosome. It is characteristic of the heterogenotes arising from high-frequency transduction that they are unstable and rapidly segregate mutant-type clones. No attempts were made to test the effect of deoxyribonuclease on this system and, therefore, since transformation has been reported in Salmonella (Hartman and Goodgal, 1959), this possibility must be entertained. However, the ease of isolation of fimbriate transductants probably excludes transformation, which is often a difficult technique to apply routinely. A heat-killed suspension of a non-lysogenic strain did not transfer fimbriation to recipient cells, so that a heat-resistant particle was not responsible. These various controls have shown that the process involved is phage-mediated transduction.

It soon emerged that the efficiency of the transduction experiments depended primarily on the successful selection of the fimbriate transductants after phage addition, i.e. the ability of the minority of fimbriate transductants to form a pellicle. Fimbriate transductants were not detected when the experiments were performed under conditions that prevent pellicle formation, i.e. by culture in anaerobic broth, shaken broth or glucose broth. On a number of occasions, attempts to inhibit the transduction process by incubating the phage-bacteria mixtures under anaerobic conditions gave a completely different effect and did, in fact, increase the transduction efficiency by about three times compared to parallel broths incubated under

aerobic static conditions. This was shown to be due to faulty anaerobic jars that could not be completely evacuated, i.e. the conditions were microaerophilic. These findings were repeatedly confirmed and investigated on a quantitative basis in the experiments described in section II. This finding is not really surprising in view of the fact that positive aerotaxis has been demonstrated in motile salmonella cells to an oxygen concentration considerably less than that present in air (Baracchini and Sherris, 1959). This phenomenon of positive aerotaxis would seem to be the real key to the requirement for motility in the recipient strain, which strictly controls the efficiency of transduction to fimbriation. This is seen from table 5 which shows that fimbriation could not be transduced into non-motile cells (including paralysed flagellates), except on a single occasion. Motility of flagellate cells is not the random process often assumed, but a definite directed response to an environment that is more favourable for the multiplication of the motile cells. Baracchini and Sherris (1959) showed that all aerobic and facultatively anaerobic motile cells possessed this ability to migrate in tubes towards the air-broth interface, and it is this which controls the success of transduction because the migration of motile cells allows fimbriate cells to establish themselves at the surface by flotation and pellicle formation. In non-motile cells, where this migration to the surface is a chance event, dependent on the convection currents in the medium, it is clear that the failure to transduce was due to an impaired

selective mechanism. Attempts to simulate positive aerotaxis by gentle shaking of the medium and by reducing the medium depth were unsuccessful.

Tests for allelism among 140 non-fimbriate strains of S.typhimurium were carried out by reciprocal transduction, which revealed that well-defined groups were present. Transduction to fimbriation was generally not detected among members of the same group, or less frequently than between members of different groups. The existence of pseudoallelism has been demonstrated wherever intensive studies have been performed to reveal this phenomenon, and this particular instance has proved no exception. The results quoted in tables 3, 7 and 8 reveal that the non-fimbriate mutants can be grouped as follows:

Group 1 - Sa 519
Group 2 - 132 FIRN strains and 1 FIRN' strain
Group 3 - Sa 749, Sa 750 and Sa 1436
Group 4 - Sa 619 and 6631
Group 5 - Sa 1566b and Dubnau strains

The original classification of the seven non-fimbriate mutants into groups A and B on the basis of their inositol-fermenting character, gave a good correlation with the grouping based on transduction studies, although slight differences emerged.

Sa 519 is obviously unique, and its inability to be transduced does not reside solely in its poor motility, since motile mutants were still not efficient recipients. This is the only reported example in the Salmonella group showing a colonial difference between fimbriate and non-fimbriate mutants. Yura(1956b) made a genetic analysis by

transduction tests among purine-requiring mutants of S.typhimurium and showed that small colony revertants (which were phenotypically like the wild-type strain) were due to a suppressor mutation. He also demonstrated that the small colony type had slower growth rates in the absence of purines than the wild-type strain, and that phage prepared on the small colony type transduced auxotrophic mutants to small colony form and wild-type phenotype. Similar small colony revertants were reported by Hartman with hisB22 mutant of S.typhimurium (see, Demerec, 1956). Loveless and Howarth (1959) also describe small colony partial revertants among a series of cys - mutants of S.typhimurium, which showed the phenotype of the wild-type. Again, phage prepared on the small colony types transduced mutants of the same group to a small colony form. This small colony size is a characteristic property of suppressor revertants, and all these examples are due to a mutation at a suppressor locus which can partially restore the wild-type phenotype in the presence of a mutant gene. It is thought that these revertants exhibit wild-type function by means of an alternative pathway. Small colony revertants are often found to be multisite mutants, i.e. they are unable to recombine with a number of mutants of the same phenotype which themselves recombine. In every respect therefore, Sa 519 conforms to the characters of a suppressor mutant and a multisite mutant - (i) the partial restoration to the wild-type phenotype is seen from the very slight haemagglutinating activity of

Sa 519 HA +, and by the scanty short fimbriae detected on a minority of cells (ii) Sa 519 did not recombine with any other non-fimbriate mutant (iii) only phage prepared on the small colony mutant transduced the parent to a haemagglutinating state.

The other three strains of group A behaved in a manner similar to one another. Sa 749 and 750 differed in that Sa 750 had a lower transduction efficiency. However, variations of this nature are more likely to be caused by physiological, rather than genetical differences in the strains. Sa 1436, although epidemiological evidence suggested that this strain was unique, behaved similarly to Sa 749 and 750. Allelic recombination to wild-type was not demonstrated by reciprocal transduction among the three strains. However, if the three strains were each mutated at individual sites all close to one another, the possibility of crossing-over and recombination occurring would be slight, and so inability to detect recombinants may reside in the limitations of the selection procedure. The detection of recombinants in crosses between FIRN and groups A and B was the first indication of complex loci in the segment controlling fimbriation.

In group B, the three strains - Sa 619, Sa 1566b and 6631 - showed differences when tested with other non-fimbriate mutants by transduction. All three were capable of recombination with the non-fimbriate strains of Group A, but only Sa 1566b gave recombinants with FIRN non-fimbriate strains. Neither Sa 619 nor 6631 was a good strain for

transduction work. These strains were transduced at a low frequency even with donor lysates from wild-type strains, and phage was propagated on them only with difficulty. The reason for this is not known and any interpretations from experiments in which these strains were used as recipients in crosses with other non-fimbriate strains must be made with caution. Sa 1566b differed from all other group A and B mutants in being both an efficient donor and an efficient recipient. The only exception to this generalisation is that recombinants were never isolated from crosses between Sa 1566b and the Dubnau strains (isolated by ethyl methane sulphonate treatment). This incompatibility between two strains, each of which is an efficient donor and recipient, suggests that the two have a common or overlapping mutation. However, one difference emerged between these two strains viz., with the FIRN strains as recipients, recombinants were detected only with lysates prepared on the Dubnau series of mutants.

Thus recombination has been detected between FIRN and group A strains and FIRN and Sa 1566b, only when FIRN strains were used as donors. Examples of mutants which are able to function as donors but not as recipients have been mentioned in the introduction, for which it was explained that the mutation involved covers a portion of the chromosome larger than that which can be carried by a single phage article. In such cases only double transduction would reveal effective recombination. Such an explanation is not valid here because the FIRN group does

function as a recipient in certain crosses so that the mutation involved in FIRN is smaller than the length of chromosome barried by P22 phage. However, the difference is probably a physiological one between the strains, and, perhaps, a statistical one because in crosses between FIRN and the other mutants, the FIRN strains were more often tested as donor than as recipient.

Thus, it has been possible to show recombination in crosses that involved the minority of strains that were rha + fim -, among each other and with FIRN. On the other hand, table 3 shows that allelic recombination to wild-type fimbriation did not occur among the 132 non-fimbriate FIRN strains at a significantly detectable rate. In experiments in which one FIRN is treated with transducing lysate from some independently isolated FIRN strain, with negative results, two hypotheses can be proposed which would account for these findings:- (a) that each of the strains is mutated at sites which are identical or over-lapping, (b) that the two strains, although not identical, are mutated at sites which are so close that the possibility of crossing-over and recombination between them during incorporation of the transduced chromosome fragment is very small, and so the yield of fim + alleles would be reduced. It is necessary to choose between these two theories to evaluate the inter-relationship of the 132 epidemiologically distinct FIRN strains. In more than 900 reciprocal crosses between 132 FIRN non-fimbriate mutants, fimbriate trans-ductants were never detected, which finding favours

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hypothesis (a). However, we have seen that recombination to fim + even among non-fimbriate mutants of the different groups is detected only with a low frequency. The likelihood of detecting fimbriate recombinants from a cross between non-identical intralocal alleles would be slight - (i) because the transduction rate for intralocal recombination is usually two or three decades lower than in crosses between defective alleles from different loci (ii) which would mean detecting a single fimbriate cell among 10^{10} cells in a broth mixture - this is beyond the limits of the broth selection technique both from a practical and a theoretical point of view. It was clear that this problem of inter-FIRN relationship could not be solved by this approach. However, if as the limited data suggest, all the FIRN strains have a common or overlapping mutation, then one would expect that a lysate from any FIRN strain would be unable to evoke a rha + transductant with any other FIRN strain as recipient. The experimental data in table 9., although incomplete since not all possible inter-FIRN crosses could be tested, suggest that this is so, because in more than 250 inter-FIRN crosses, no rha + transductant was ever detected. In this case, with an absolute selective medium and a high transduction rate, any rare recombinants should have been detected with ease even if formed at a rate 0.1% less than when a wild-type rhamnose-utilising donor was used.

The cumulative experimental evidence is convincing enough to show that no FIRN strain was ever transduced to

rha + fim + in a single-step transduction, regardless of how the transductant type was primarily selected. The high correlation of rha and fim characters in the naturally isolated Salmonella typhimurium strains is, therefore, not explained by a genetic linkage. This has been verified by Subbiah and Stocker (1962) who used colicinogenic agents as fertility factors in S.typhimurium and showed, by analysis of recombinant markers, that rha was between metA and str, and that fim, although not definitely located, did not map between these two characters. Not even a physiological linkage can account for this correlation of two apparently unrelated characters because, although all the fimbriate transductants tested were rhamnose non-fermenting, their behaviour in artificial culture was in every way comparable to the naturally isolated FIRP strains. If rhamnose is, in some way, necessary for the synthesis of fimbriae, it must be synthesised by some alternative pathway, not only in these transductants but also in the naturally occurring fim+rha- strains (Sa 1179, Sa 1180, Sa 1541, 6768 and 6899) of S.typhimurium and in other Salmonella species in which there is no correlation of the two characters. How, then, can this all-or-none correlation of markers in S.typhimurium natural isolates be explained? The most obvious theory, and one which also accounts for the absence of inter-FIRN fertility in crosses to both rha + and fim +, is that these 132 FIRN strains are, in fact, representative of the repeated isolation of the one FIRN type. This is but another expression of hypothesis (a), that all FIRN strains

have identical or overlapping mutations in both characters, and that they have evolved from a common parent, which was rha - fim -. Neither the genetical nor the epidemiological evidence enables one to decide (i) whether the present-day FIRN strains represent direct progeny of this hypothetical parent, i.e. have the same mutations in these two characters as the original parent or (ii) whether several different lines were originally mutated at individual sites in fim and rha, and that during the course of their spread, many subsequent mutations were accumulated, so that genetic similarity in the FIRN strains is due to a common overlapping mutation of considerable length, i.e. a multisite mutant. The fact that FIRN strains exhibited a high transduction efficiency and reverted to fimbriation on treatment with mutagens suggest that the mutation involved in FIRN is not extensive, and indirectly favours hypothesis (i) above.

The conclusion that these FIRN strains represent the one type of S.typhimurium infers that the FIRN group is a very successful world-wide type which, despite its mutations in rha and fim, has established itself to diverse animal hosts. The differences in some of the strains, such as loss of flagellation, are readily explained by mutations that have arisen in a minority of strains. The acquisition by only certain FIRN strains of the ability to produce colicins is readily explained since we have seen that this property is controlled by episomes that are transferred often at high frequency and also irreversibly lost. The range of phage-types among the FIRN strains may

be explained likewise. The different phage types, although a factor of great importance to the epidemiologist, become insignificant as a criterion for genetic diversity in the non-fimbriate FIRN mutants. It is, however, necessary to propose that the hypothetical parent was non-lysogenic, and that different phage-types originated by subsequent phage infection during their spread. It is interesting to note that a few of the FIRN strains are non-lysogenic.

Theoretically it should be possible to determine the order of the various mutations in the non-fimbriate classes, and so present a genetic map of this section of the chromosome. One simple method is to arrange the sites according to the frequency with which the markers recombine, assuming that the frequency of recombination is proportional to the distance between markers. However, although this method is useful and frequently used, it cannot be applied in these studies because of the obvious variability in the mutants available. For example, this criterion could not be applied to the groups A,B and FIRN because their transduction efficiencies differed so greatly from strain to strain even with wild-type donor lysates, that attempting to order the different sites from consideration of the frequency of recombinants between the different classes would be of no significance.

Another method which has been used to map the order of mutations among a series of mutants is by crosses between a multisite mutant and the different markers concerned. Failure to detect recombination is indicative

that the multisite mutation overlaps the mutation of the particular mutant tested (Hartman, Hartman and Serman,1960a). Sa 519, although apparently a multisite mutant, gives no help because it failed to recombine with any other of the non-fimbriate groups and this particular mutant therefore, must overlap the mutation of all the other non-fimbriate strains examined.

Yet another method that has been used to map the order of sites on the chromosome is abortive transduction (Ozeki,1956, Hartman et al, 1960a). Abortive transductants are usually recognised by their weak prototroph state on selective media (see also Stocker et al, 1953). However, it is impossible to recognise an abortive fimbriate transductant, because one can only refer to their specific properties of agglutination and pellicle formation in broth, and impaired activity in both these properties can come about by factors other than abortive transduction.

Thus, the obvious strain differences among a limited number of non-fimbriate mutants and the fact that the available methods do not allow satisfactory quantitative assessment, mean that the construction of a genetic map for this region must rest with the origin of an improved selective medium or indicator system for fimbriate cells. It is also impossible to discuss the mutations in these groups of mutants in terms of specific deficiencies in the synthesis of fimbriae. No doubt this will come with a more detailed knowledge of the structure of fimbriae, an

improved selection mechanism and a larger number of non-fimbriate mutants, derived artificially by the use of mutagens of known activity.

In addition, the inability to examine possible differences in the morphology of abnormal fimbriae except by screening large numbers of cells by electron microscopy will probably continue to control the progress of this particular aspect of fimbriation,. Nevertheless, Duguid, (unpublished results), has observed abnormal fimbriae in a few cells of Klebsiella ozaenae. These morphologically-altered structures were about 4 - 40 in number per cell, of the same thickness as fimbriae but of length normally associated with flagella. Investigations on the genetic control and the chemical structure of such abnormal "long" fimbriae would obviously be invaluable, but, again, impossible at this stage. No doubt, the many gaps will be filled and the present problems solved as this particular study progresses into a more advanced stage. It must be remembered that the study of fimbriation is still in its infancy when compared to the study of flagellation.

Mutagenic response.

One interesting fact that emerged from the discovery of non-identical allelism in bacteria was that the non-identical alleles often showed different mutation patterns with respect to their behaviour on treatment with mutagens. For example, sixty per cent. of S.typhimurium mutants are mutagen stable (Hartman, 1956). Because it seemed to offer a further means of solving the relationship between

the different non-fimbriate types, a short study was made on the mutagen stability of these mutants. The results outlined in section I show that among the seven non-fimbriate mutants of groups A and B, only three could be induced to revert to the fimbriate state - viz., Sa 749, 1436 and 1566b - i.e. 60% of the mutants were mutagen stable. Here, although the epidemiological and transduction data have indicated that Sa 749 and Sa 750 were the same isolate, we note the anomaly of the behaviour of Sa 750 when treated with mutagens. The differential behaviour among the seven non-fimbriate mutants of groups A and B confirms indirectly the finding of non-identical allelism. With the non-fimbriate FIRN strains, the results were initially most favourable. Limited tests on all the FIRN strains had revealed that they did not revert to the fimbriate state, when treated with manganous chloride, ultraviolet light or ethyl methane sulphonate. This finding of consistently negative results, i.e. mutagen stability among the FIRN strains, fitted in well with the transduction data - i.e.with the hypothesis that they showed an identical or overlapping mutation. Further testing, however, revealed that six fimbriate revertants were obtained from the 132 FIRN and 1 FIRN' strains, five with manganous chloride and one with ultraviolet light. The identity of these strains as FIRN derivatives was undeniable, and the fimbriae examined by electron microscopy quite normal. No FIRN strain ever reverted on more than one occasion, and attempts to confirm

a positive result for any FIRN revertant by repeated testing (up to 24 times) of that same strain failed to give verification of the mutagen lability of that strain. The conclusion that this difference in mutagen behaviour is indicative of non-identical allelism among the FIRN strains is unfair because of its contradiction of the more detailed genetic analysis and because of the irregular origin of the FIRN revertants.

Not too much significance must be placed on the apparent finding that manganous chloride was the most efficient mutagen, because it was tested approximately six times more often than u.v. irradiation, and ethyl methane sulphonate was tested on only a chosen twelve strains. Certainly, if manganous chloride were a more efficient mutagen than ultraviolet irradiation, this would be surprising because it is a generally accepted rule that no mutagen-labile mutant is known that is resistant to ultra-violet irradiation (see Demerec and Hartman, 1959).

One interesting finding that emerges from the work with manganous chloride is that the revertant fimbriate cells from groups A and B were detected more readily than those from FIRN. This could be explained (i) by a greater killing of the group A and B strains, which would mean that any small minority of revertants formed would have a better chance of outgrowing the survivors of non-fimbriate status or (ii) that the mutation involved in the groups A and B is smaller and more readily corrected than that in the FIRN group. Control counts did not indicate a

differential killing. If the FIRN strains, after mutagen treatment, were only partially restored to the wild-type genotype, the the slow origin of fimbriate FIRN revertants (sometimes as many as three 48 hr. subcultures) can be explained by the hypothesis that it was not restored to full wild-type but to a state from which secondary spontaneous mutants might arise. The fact that fimbriate revertants were never obtained from the Dubnau strain is not surprising since they were derived from S.typhimurium LT-2, a line in which it is difficult to induce reversions (Demerec et al, 1958).

It must be qualified here that by mutagen lability, we are referring to the fact that all the non-fimbriate strains that reverted never showed spontaneous mutation to the fimbriate state. The observations made that spontaneous mutation never occurred agree with those of Duguid (unpublished results). Although it is characteristic of multisite mutants that they do not revert, it is quite clear that the FIRN strains are not multisite. Failure to revert does not, of course, necessarily mean a long deletion (i.e. multipoint deletion), because a short deletion can be equally stable, and, in fact, certain mutants which are presumed single site mutants are quite stable (Hartman, Loper and Serman, 1960b). The stability to spontaneous mutation of all the non-fimbriate strains, is, therefore, not in any way indicative of the length of the mutations involved.

These are the first known experiments on the effects of mutagens on non-fimbriate mutants, and although only a preliminary survey has been made, the results were interesting. They justify a further investigation in detail on the effect of induced mutation on both fimbriate and non-fimbriate strains which, unfortunately, it was not possible to attempt. It is possible that if the FIRN strains were tested often enough and with a wide range of mutagens under conditions better established for the selection of fimbriate revertants, all the FIRN strains would revert to fimbriate status and add weight to the hypothesis that they are the same strain genetically.

Phase variation.

Since the reversible fim + $\rightleftharpoons$ fim (+) phase variation occurs at the very high rate of 10^{-3} per bacterium per generation, (Brinton et al, 1954), it seems unlikely that it is a result of mutation (i.e. alteration, arrangement or loss of genes) and more likely to involve a controlling mechanism of episomic nature. Presumably, such an episome was implied when it was suggested that fimbrial phase variation depended on spontaneous changes in a heritable determinant (Duguid and Wilkinson, 1961). It is true that, in some obvious ways, fimbriae qualify for consideration as cell processes that are subject to episomal control (i) because they are non-essential structures (ii) which can be irreversibly lost without damage to the cell and (iii) because their presence on a bacterium is, in most cases, an alternately expressed character. A single episome can be responsible

for three different phenotypes in a cell according to whether it is integrated or autonomous or absent (Jacob, Schaeffer and Wollman, 1960). Such a system could readily be envisaged which would account for the fimbrial phase variation in Salmonella typhimurium, the fim * ⇌ fim (+) variation being determined by the alternating integrated and cytoplasmic locations of the hypothetical episome determining synthesis of fimbriae, which would be irreversibly lost in the fim - type cell.

Fimbriate S.typhimurium strains were examined for the presence of such an episome by consideration of two properties common to many episomes - their sensitivity to the acridine dyes and their transmission from cell-to-cell at high frequency. The role of the fim episome would presumably be to co-ordinate with the structural fim gene in the determination of the synthesis of fimbriae, and the differences in the two phases would represent the alternate sites of the fim episome. Since the synthesis of fimbriae might have been dependent on either the autonomous or integrated position of the episome, the experiments in section I were devised so that both phases were subjected to acridine treatment. Neither method disinfected the cell (i.e. removed its ability to synthesis fimbriae) and so, if an episome controls this variation, it is not an acriflavine-sensitive one. The speed with which fimbriate cells dominate a population under certain conditions (see Section II of results) was suggestive of a cell-to-cell infection. All attempts to transfer fimbriation from a typical phase-

varying Salmonella typhimurium to a permanently non-fimbriate one were unsuccessful. Therefore, inability to synthesise fimbriae by fim - cells is not because they have irreversibly lost the co-ordinating fim episome, but because of mutations in the structural gene fim.

However, although we have failed to establish the presence of an episome by the absence of these two indicative episomic criteria, any interpretations must be made cautiously on three accounts (i) only a limited number of strains has been tested (ii) acridine-resistant episomes have previously been described (Holloway and Fargie,1960) and (iii) ability to transmit colicins at high frequency in S.typhimurium strains is a property only of newly-infected cells, which is lost after three to seven generations of growth (Smith and Stocker, 1962). If transmissibility of the hypothetical fim episome was also dependent on recent infection, then the negative results would be explained - because the strains examined were all laboratory cultures of some age.

Episomes with properties intermediate to, or completely different from, the classically described episomes will, no doubt, be discovered in time, and it is, as yet, premature to dismiss the existence of a fim episome on the available limited data. However, one may echo Jacob, Schaeffer and Wollman (1960) when they said that "the danger of episomes is that they may provide a model capable of explaining too many things.....and to invoke the presence of such elements to account for complex situations

of nucleo-cytoplasmic inter-relations".

Maccacaro and Hayes (1961a) concluded that the variation of fimbriate types was complex, and attempted to explain the five different types in E.coli - fim σ+, fim +, fim (+), fim σ= and fim - - by a model dependent on a structural gene and an episome (controlling the type of fimbriation), from which it was necessarily concluded that the difference between a fim + and a fim σ= cell resided in the absence of an episome from the latter. Failure to transfer fimbriation from fim + cells to fim σ= cells, either by contact or conjugal infection (excluding transfer of the fim locus) caused them to discard this theory. Brinton (personal communication) has also failed to "cure" fimbriation in E.coli cells by disinfection with acridine, and to transfer fimbriation by cell contact. The existence of a fim episome in E.coli or S.typhimurium is, therefore, hypothetical.

Maccacaro and Hayes (1961) found evidence of a second genetic determinant controlling fimbriation in E.coli. This was a chromosomal locus σ which could be separated from fim occasionally by transduction but never by recombination. Its presence resulted in the stability of the permanently fimbriate fim σ+ type. Mutations in this locus were apparently responsible for the fim σ- type which mutated from the fim σ+. The involvement of a second chromosomal determinant controlling the fim + to fim σ+ variation is quite acceptable because it does not demand rapid mutations in the σ locus, since the variation is generally

irreversible. As mentioned earlier, variations at a second chromosomal locus will probably not explain the reversible and rapid fim + ⇌ fim (+) variation. However, this variation, found in E.coli, Sh.flexneri, Salmonella and Proteus hauseri strains, could be explained by the assumption that the synthesis of fimbriae, like the synthesis of other molecular species, is controlled by regulator genes producing specific repressors which block the activity of the fimbriation operon. The presence or absence of this hypothetical repressor will determine whether a genotypically fimbriate cell is phenotypically non-fimbriate or fimbriate. A further extension of the theory would account for the fim σ+ and fim σ- types as caused by mutations in the regulator gene and manifested by a permanent inability to switch off or switch on the operon (the fim σ- type may also have structural gene mutations). The fim- type would be the result of mutations in the structural fim gene. The regulator locus would presumably be near-sited to the operator gene, and might even be the σ locus of Maccacaro and Hayes (1961a).

Acceptance of this control-by-repressor theory means that under conditions where fimbriate cells can form a pellicle, the repressor system is absent and the fimbriae are synthesised, and conversely for the conditions unfavourable to fimbriation. This would suggest that the environmental conditions (as well as selecting the variants) do, in fact, exert a direct effect on the synthesis of fimbriae by the induction of the repressor, a suggestion in contradiction

to that by Duguid and Gillies (1957). They suggested that the environmental conditions were important only in the selection of the variants, a consideration based on the fact that since Shigella flexneri strains were converted to a new phase sometimes only after three or four cultivations under the inducing conditions, this could not be a direct effect of the environment since it was so slow. However, Shigella flexneri is the exception among the phase-varying fimbriate enterobacteria - Salmonella, E.coli and recently isolated Proteus hauseri strains usually dominate aerobic static broth cultures within 24-48 hr. Admittedly this is slower than some environmentally-induced changes, e.g.volutin production (Smith, Duguid and Wilkinson, 1954), but partial explanations can account for this. Firstly, if one accepts that the main role of fimbriae is one of survival and not of nutrition, then it is clear that there will be no need for the synthesis of fimbriae until the later stages of growth, and the rate of change on transfer from agar to broth not significant. Secondly, the slow speed at which repression and derepression occur could be explained by an inaccessibility of the repressor to the site of fimbrial synthesis. The origin of fimbriae must be within the cytoplasmic membrane, because Maccacaro and Turri (1959b) discuss fimbriate spheroplasts and also, electron micrographs suggest that the fimbriae penetrate the cell wall (see figure 1.). Although there is no electron microscopical evidence of a basal granule for fimbriae it is convenient to argue that such a granule might represent the site of fimbrial protein

synthesis. Synthesis of fimbriae is not inhibited by chloramphenicol (Brinton, personal communication) which suggests either that the synthesis of this particular protein is in some way unusual or that the site of fimbrial synthesis is inaccessible to the chloramphenicol, the latter finding fitting with the theory that the hypothetical granules (presumably just beneath the cell membrane) might be responsible for synthesis. If so, these sites are quite removed from the general cytoplasm and, hence, inaccessible. This might then explain why the repression and derepression of fimbrial synthesis is slower in being effected and why this variation is not as readily impressed by cultural conditions as other phenotypic variations. This mechanism of repressor control is, of course, completely hypothetical.

Transduction of haemagglutinating ability.

Exhaustive screening by electron microscopy has revealed a minority of fimbriate strains that are completely lacking in haemagglutinating activity. As well as their occurrence in certain Salmonella paratyphi B strains, they have also been observed, commonly, in Salmonella gallinarum and Salmonella pullorum strains,(Duguid, unpublished results). Analogous to the presence of the gene mot, controlling function in flagellated Salmonella strains (Stocker et al, 1953), attempts were made to provide evidence for the existence of a gene Ha controlling the haemagglutinating ability of fimbriae. The S.paratyphi strains with non-functional fimbriae were transduced to Ha + only with

lysates propagated on wild-type fimbriate S.typhimurium donors. The necessity for this Ha factor for flotation is obvious, otherwise the transductants with active fimbriae would not have been selected from the broth mixture. The parallel growth curves in fig.2. show that the greater growth level attained in aerobic static broth by the form with functional fimbriae was accompanied by the formation of a fimbrial pellicle. These growth curves show a great resemblance to those obtained with fimbriate and non-fimbriate strains of Shigella flexneri (Duguid and Wilkinson, 1961), suggesting that the presence of inactive fimbriae on a cell causes it to behave as a non-fimbriate strain. The agglutination of red cells and other substrates by fimbriate organisms is thought to be dependent on the presence of special groups on the surface of the fimbriae, which are complemented by a site common to the surface of a variety of cell surfaces. The inability of non-functional fimbriae to cause agglutination may, therefore, reside in an altered chemical structure in these fimbriae, which causes a masking of the key groups on the polypeptide chain. In the light of what we now know of the chemistry of fimbriae, it would be most interesting to isolate functional and non-functional fimbriae and see if there was any difference in the chemistry, quantitative or qualitative, of the normal protein of S.paratyphi B, Sa 66 Ha+ and the abnormal protein from Sa 66 Ha-. So far, the only literature on these non-functional fimbriae is presented by Campbell (1961) who found that the fimbriae of

S.pullorum and S.gallinarum had the fimbrial antigens 1 and 5. The flagella from motile and paralysed strains of S.typhimurium showed no serological differences in cross-absorption tests (Iino, 1958b). It is possible that the differences in the two kinds of fimbriae will be so minor that they will not be differentiated serologically.

However, it must be admitted that the evidence for the existence of this Ha gene is, as yet, incomplete. Certainly, since the function of fimbriae (unlike that of flagella) apparently depends solely on their surface configuration, it is not clear that a distinct unit of function Ha other than the structural fim gene controlling fimbrial synthesis, will be necessary to effect activity. However, if a gene Ha has not been transduced, the alternative conclusion is that the whole segment of the chromosome controlling the synthesis of fimbriae has been transduced, i.e. the transduced strains of S.paratyphi B now synthesise functional "typhimurium" fimbriae and not "paratyphi B" fimbriae. Unless the hypothetical Ha factor is itself antigenic, it would be impossible to disprove this theory with the two strains used in the crosses, because both S.typhimurium and (normal) S.paratyphi B strains have the same fimbrial antigens, 1,2 and 3 (Campbell, 1961). This difficulty, however, could be resolved by transducing haemagglutinating activity from a donor strain with different fimbrial antigens from S.paratyphi B. Cross-absorption tests with purified fimbriae from the donor and the transductant with haemagglutinating fimbriae would reveal any

differences in the two, and show whether the fimbriae of the transductant were, serologically, those of the donor or the overt "paratyphi B" antigens.

Co-transduction of fim and fla into the Dubnau line.

Since transduction transfers such a limited portion of the donor genome, it was hoped that, by the detection of certain classes of cotransductants, fim might be located on the chromosome. The hypotheses of close genetic linkage of fim and rha or inl were not valid since such cotransductants were never detected. Since a few fimbriate transductants, screened randomly, were found to be inositol-fermenting, this must have been due to the origin of spontaneous inositol-fermenting mutants. Maccacaro and Hayes (1961a) were also unable to locate fim on the chromosome of E.coli by the isolation of cotransductants.

However, one interesting example of cotransduction was found - when strain Sa 1137 was treated with lysate from the wild-type fimbriate S.typhimurium strain, SL 497, and selected for fla +, a small number of fim+fla+ cotransductants were detected at a low frequency. This close linkage of fim and fla was never shown when other non-motile, non-fimbriate S.typhimurium strains of FIRN class were transduced to either fim or fla.+. Admittedly, this Dubnau non-fimbriate line is unusual in other ways e.g. its transduction efficiency as a donor with the non-fimbriate FIRN strains was higher than even wild-type fimbriate donors. However, indication of a close linkage of fim and fla also comes from the fact that of the five Dubnau recombinants selected for fla + by Miss Sylvia Smith (see page 110),

only one was stable in its fim - character, suggesting that recombination had introduced both fim and fla. This phenomenon came to light only a short while before the completion of this work, and the problem has not been analysed in detail. Therefore, it is not yet clear whether this is a genuine example of cotransduction or if some other explanation can be advanced.

Competition experiments.

The experimental results presented in Section II dealt with competition experiments, and these will now be discussed not only with reference to their verification of the efficiency of the transduction procedure, but also with respect to a possible role for fimbriae, particularly in saprophytic organisms. These results show that when small numbers of naturally isolated fimbriate strains were grown in competition with large numbers of naturally-isolated non-fimbriate strains, the fimbriate strain outgrew the non-fimbriate strains within a 48 hr. period if the broths were incubated aerobically or microaerophilically and statically i.e. under conditions which favour pellicle formation. This increase was usually observed in the second 24 hr. period of mixed culture, but in some experiments the fimbriate population was considerable even after 24 hr. The extent of outgrowth varied from experiment to experiment, but usually represented a relative increase of fimbriate cells of several million times. There appeared to be no limiting level of challenger organisms beyond which competition could not occur; as few as 1 - 3 fimbriate cells were capable of outgrowing non-fimbriate cells (see experiment 4,

and Contento and Old, unpublished results). This outgrowth was always accompanied by the formation of a pellicle at the broth surface and an increase in the haemagglutinating power, so that all three phenomena seemed related. Under conditions which did not allow the formation of a fimbrial pellicle, the fimbriate cells were not greatly favoured, except that, in some cases, the naturally isolated fimbriate strains (Sa 206 and LT-2) of S.typhimurium increased less significantly than under aerobic static conditions,i.e. in rotated or shaken broths, or on agar plates. This increase was observed even when buffered media were used so that the increase did not represent a differential killing of the non-fimbriate cells by the low pH produced towards the end of the 48 hr. period. It was possible that unrecognised strain differences might have accounted for the outgrowth. For example, the strains Sa 206 and LT-2 might have different metabolic activities from the non-fimbriate strains, and these differences, not related to fimbriation except coincidentally, might have allowed their greater proliferation in broth cultures shaken artificially. Alternately, the naturally isolated fimbriate strains might have possessed a greater resistance to the accumulated toxic waste products in the medium, or might have been less liable to autolysis than the non-fimbriate strains examined. Thus, although these experiments demonstrated the efficiency of selection of the fimbriate cells under conducive conditions to pellicle formation, it was not possible to show parallel

control experiments in which outgrowth was prevented by inhibition of this ability. In order to overcome the possible strain differences inherent in naturally-isolated strains, further experiments used fimbriate and non-fimbriate strains derived from the same parent by transduction, so that the restricted transfer of genetic material ensures that the only difference between such strains is the one transduced viz. fimbriation. With a fimbriate and a non-fimbriate pair derived from S.typhimurium FIRN strain Sa 625, we see that under conditions favourable to the formation of a pellicle, the small numbers of fimbriate cells again outgrew the large numbers of challenged non-fimbriate strain. Contrary to the experiments using naturally-isolated strains, there was no outgrowth, i.e. no selection of the fimbriate member of the pair, when pellicle formation was prevented by shaking etc. These findings were confirmed in experiments with another pair from FIRN Sa 635. All possible controls have been included to validate the findings of these experiments and show that they are not carefully reproduced artefacts. For instance, prior to every experiment, the challenger and challenged strains were cross-tested to show that neither produced colicin or phage lethal to the other, so that outgrowth by the fimbriate cell was not due to its ability to kill the other. The experiments were also constructed so that, at the time of challenge, the two participants were, as far as possible, in the same physiological state since they had been subjected to the same environmental

conditions. The literature on fimbriation would seem already to be confused by failure to ensure that fimbriate and non-fimbriate cells for comparative purposes are physiologically similar. In the introduction, it was noted that non-fimbriate cells (of E.coli B) had a shorter generation time at 37° than fimbriate cells, which would eventually lead to the elimination of the fimbriate type in the absence of some compensatory mechanism. It is suggested that these experiments are proof of the compensatory role of fimbriae and pellicle formation.

The outgrowth by the fimbriate cells in such a short time (e.g. table 19 shows that 30% of the cells were fimbriate after 24 hr. representing a relative increase of the fimbriate cells of 125,000-fold) is exceptionally efficient and brings to mind the rapid transmission and epidemic infection of a population by the determinants called episomes. For example, when F+ cells of E.coli are mixed with F- cells, up to 50% of the latter acquire the F+ factor within a short period of an hour (Cavalli, Lederberg and Lederberg, 1953). Transmissibility of the colicinogenic factors also occurs by cell-to-cell infection - experiments showed that when stock colI. and col.- lines were incubated together for 2 hr. in the ratio of 1:20, 40% of the cells were col.I +, and it was concluded that a minority of the col.I cells could initiate the epidemic, because the episome replicated at a rate faster than the chromosome (see Smith and Stocker, 1962). Nakaya et al (1960) showed that resistance transfer (by episome) between

E.coli and Shigella occurred at a high frequency, being almost complete after 2 hr. The possibility had to be entertained that the outgrowth of fimbriate cells might be due to episomic infection in the early stages of mixed culture and the subsequent selection of the fimbriate type. In the experiments quoted, each fimbriate cell was marked as rha+ and each non-fimbriate cell as rha- to allow differential counting at chosen intervals. Small numbers of rha+ and rha- colonies from the final population in every experiment were randomly selected and screened. In this series of experiments, more than 300 single colonies were checked, and the two characters remained associated without exception, i.e. no hybrid fim+rha- type was obtained. Thus, unless the two unrelated characters rha and fim are carried by a single episome, in a manner analogous to the four closely related factors giving multiple drug resistance, it would seem that episomic infection can be dismissed. The competition experiments with S.typhimurium LT-2 and Sa 7471 are invaluable in this argument, because these strains differed in six characters. Large numbers of randomly screened colonies had the correct identity of either the LT-2 or Sa 7471 strain, showing that the outgrowth by LT-2 was due to the multiplication and selection of the originally injected challenger cells. It would also have been difficult to explain why, if this increase was episomically controlled, the transmission had occurred only under aerobic static conditions and not in shaken or rotated broths or in glucose static broths (Contento and Old, unpublished results).

Experiments 1 - 7, therefore, may be considered verification of serial broth subculture as a satisfactory selective medium for the detection of fimbriate transductants. Although the transduction rate, as calculated in but two experiments, is low, with probably only ten cells transduced per experiment, this small number of cells in a transduction experiment is capable of being selected, if the results from experiment 4 are accepted. The experiments outlined in tables 20 and 21 were intended as reconstructions of the transduction procedure for non-motile FIRN recipients. The absence of competition using a phenotypically non-fimbriate cell as challenger is in agreement with the large number of negative results in experiments in which non-motile cells were used as recipients. It has been suggested that this inability of fimbriate cells to be selected is because they are not transported to the air-broth interface in the absence of positive aerotaxis which is dependent on cell motility (Baracchini and Sherris, 1959). The small number of potentially fimbriate cells in such an instance cannot divide rapidly enough because of their longer generation times and are killed off by the exhaustion of the medium by the non-fimbriate culture.

In a subsequent experiment, where both the challenger (again *fim*+*fla*-) and the challenged (*fim*-*fla*-) strains were subcultured through three broth subcultures, the fimbriate cells were selected between 48 and 96 hr., apparently because fimbriation was phenotypically expressed in this challenger. However, in a limited number of

transduction experiments with non-motile FIRN recipients, incubation was continued for two one-week subcultures without any additional success in the selection of the hypothetical fim+ transductants. The difficulty in transducing non-motile cells would seem, therefore, to be partly due to the lag period, after phage addition, before the effect of the incorporated fim fragment is manifested in the recipient cell, in which time the lysogenic non-fimbriate survivors dominate the broth culture, and partly due to the absence of positive aerotaxis.

The importance of flagellation alone to a cell was examined in two experiments (9 and 10). These show that the ability to form a flagellar pellicle at the surface of a static broth culture is important to the survival and multiplication of an organism in a favourable environment. The competitive outgrowth, though less dramatic than that caused by the presence of fimbriae, is significant. In the single control experiment, it was shown that the shaking nullified this advantage either by shaking off the flagella or by inhibiting pellicle formation. There are, as far as can be ascertained from the literature, no comparable experiments relevant to this ability in flagellate cells, apart from the experiments of Baracchini and Sherris (1959) which, whilst showing that motile cells aggregated at a distance some 4 mm. from the broth surface, do not mention pellicle formation.

The final competition experiments were constructed using pairs of S.typhimurium which possessed one, but not

both, surface appendages, using each in turn as challenger. Summarily, we can say that in experiment II the phenotypically fimbriate challenger strain outgrew the flagellate challenged cell because the latter did not form a flagellar pellicle, i.e. it behaved as if it had no appendages at all. The final experiment of this series showed an apparently contradictory picture. In this instance, the same fla + line as used in the previous experiment formed an extensive granular, flagellar pellicle and at 48 hr. formed 3% of the final population. It seems that in this case the fla+ line had dominated the early stages of growth by its pellicle-forming ability, but that the fim+ line was about to dominate the later stages of growth by its even-stronger pellicle-forming activities. These findings, however, are difficult to interpret, because of the variability from experiment to experiment in the ability of the same colony line to form a pellicle, whether flagellar or fimbrial.

Throughout this investigation with S.typhimurium strains, successful transduction and competition experiments have always been correlated with the formation of a fimbrial pellicle, which, in turn, has been dependent on the possession of flagella. The literature reveals that two functions have been proposed for fimbriae. Firstly, that fimbriae are important in the metabolic activities of a cell. However, the experimental data supporting this claim (Maccacaro and Dettori, 1959) has been singularly lacking in confirmation by other workers (Downie, unpublished results, Wohlheiter et al, 1962). It is difficult to envisage how fimbriate

would fulfil a nutritive role in an organism unless they can be imagined as specialised sites of permease activity. However, the known site of permease activity is the cytoplasmic membrane, and fimbriae, since they are protein structures, have a different chemical composition from the lipoprotein membrane. It would also be difficult to explain their limited species distribution and their synthesis under only a few environmental conditions when permease activity would be desirable under most conditions. The alternate theory, that fimbriae are organs whose function is one of survival by virtue of their pellicle-forming ability, seems now to have confirmation from the competition experiments. Further evidence accumulated from the growth experiments between S.paratyphi B strains with functional and non-functional fimbriae (see figure 2) when it was seen how added potential for greater growth levels and survival was attained by the gain of the Ha factor responsible for flotation. No doubt, these experiments, in artificial culture with organisms that are primarily pathogenic, will prove repeatable for saprophytic organisms, generally considered more likely to benefit from pellicle formation. A third hypothesis has been proposed concerning the function of fimbriae (Duguid et al, 1955), namely that of adherence to cell and other surfaces. Certainly, the fimbriae in typical pathogens such as Shigella flexneri and Salmonella typhi will not function through pellicle formation on stagnant liquid, since these organisms are not found in such conditions in nature.

The formation of fimbriae primarily in the later periods of exponential growth agrees with a survival role, and, of course, optimal pellicle formation occurs under the two conditions which are likely to be most common in the natural habitats favoured by saprophytic organisms, viz.static aerobic and microaerophilic environments. The inability to form a pellicle in vigorously shaken or rotated broths is not surprising, since such habitats are probably encountered nowhere other than the laboratory. The inhibition of pellicle formation by glucose (Maccacaro and Dettori,1959, and Duguid and Gillies, 1957), is likely to be a non-specific effect due to a fall in pH during growth in unbuffered glucose broth. Certainly, although these results do not disprove the nutritive role of fimbriae, they do favour the hypothesis that these organs establish an organism in an environment favourable for its multiplication and survival. An organism would beem to be maximally suited for survival by the possession of flagella and fimbriae, the former responsible for the positive aerotactic migration which assists the pellicle formation by the latter. Similar experiments are obviously desirable with other genera.

Chemistry.

The results reported in section III of this thesis confirm the conclusion that fimbriae from E.coli are pure protein (Brinton and Stone, 1961, Brinton, 1963, personal communication). The main aim ofthe purification technique reported - the isolation of fimbriae without subjecting them to extremes of pH - was fulfilled. Apprehension that the

extremes of pH used by other authors might cause a disaggregation of the fimbriae and the loss of some constituents in the process were, therefore, unfounded. Thus, there are now two methods available for the purification of fimbriae - isoelectric precipitation and column chromatography, each giving similar results. The final preparations of fimbriae have been judged pure by several criteria - electron microscopy, ultraviolet spectrophotometry, chemical analyses, agar gel diffusion and biological activity. While it would be presumptuous to consider any single property on its own as a valid criterion, for the homogeneity of the preparations, cumulatively, these provide sufficient evidence of purity.

1. Electron microscopy. Fimbriae are structures which have a characteristic appearance when examined by the electron microscope. However, while it is essential as a tool for the preliminary investigations of all preparations, its limits as a criterion of purity are obvious. The inability to detect small amounts of contaminating material - cell wall, cytoplasmic membrane or even whole bacteria - except by the laborious screening of large numbers of fields, constitutes the main drawback to electron microscopy, and, of course, it cannot detect non-particulate contaminating material. Even with preparations judged pure by several criteria, it was difficult to differentiate between the possible presence of contaminating material and the irregular granularity of the supporting membrane at high magnification.

2. Absorption spectra. The figures 10 and 11 show the absorption spectra of a crude fimbrial preparation and peaks i,ii and iii, obtained after fractionation on DEAE-cellulose. The two peaks showing haemagglutination after purification gave absorption spectra characteristic of proteins. The main contaminating material was obviously due to nucleic acid and, possibly, small amounts of spheroidal surface material not removed by differential centrifugation, because of similarity of size to the fimbriae. The spectra are useful criteria but not completely satisfactory since peak ii, judged/by this means, gave two bands of precipitation on pure gel diffusion. Continuous spectra are, unfortunately, also somewhat difficult to obtain.

3. Chemical analysis. The constant increase in the nitrogen-dry weight content at the different stages of purification was indicative that the final product would contain mainly protein. Crude fimbrial preparations, even after differential centrifugation, contained significant amounts of one or all of several products which indicated contamination of the fimbriae - phosphorus, nucleic acid and carbohydrate. These were absent from a purified preparation. The real disadvantage of this criterion was that accurate dry weights, which were performed in duplicate, demanded between 7 and 10 mg. of purified preparation per dry weight sample. The Lowry test for protein was not used since its applicability depends on the presence of certain amino acids (tryptophane and tyrosine) and it was not known whether these amino acids were present.

4. Agar gel diffusion. Crude, partially purified

and purified preparations of fimbriae were examined for homogeneity by agar gel diffusion tests. The figures 14a - 16 in section III have shown that the haemagglutinating peak i was consistently homogeneous and displayed only one precipitation band. This is a useful criterion of purity, especially since different dilutions of antigen and antiserum did not reveal further bands.

5. Biological activity. An additional criterion, although one that could not be satisfactorily put on a quantitative basis, was the fact that purified fimbrial preparations retained haemagglutinating activity. This confirmed the early suggestins of Duguid et al, (1955), who correlated the presence of fimbriae with haemagglutinating activity in E.coli strains.

Electrophoresis and ultracentrifugation are additional criteria which are often used in the characterisation of a protein. Electrophoresis depends on the surface charge on a particle (relative to its volume) and, because of the wide range of substrates for adsorption by fimbriae, any small amount of contaminating material could easily migrate with the fimbriae over a wide range of pH and not be detected. Ultracentrifugation, since its efficiency depends on size, was not thought to be applicable since the many different sizes of fimbriae would give a broad band rather than a distinct peak, so that other bands of minor contaminating material would be masked.

The chemical analysis of fimbriae as protein structures, with a typical range of amino acids, therefore,

eliminates any possible relationship between fimbriae and cell wall or capsular material, and confirms the findings of Gillies and Duguid (1958), who found that antiserum prepared against a non-fimbriate strain did not agglutinate a fimbriate organism. In this case, there can be no possible confusion between fimbriae and flagella, since a permanently non-flagellate strain was used. In addition, there are other differences between the two proteins, the one most commonly reported being the stability of fimbriae of various species to low pH's, which cause disaggregation of the flagella (Weibull and Hedvall 1953, Gillies and Duguid, 1958, and Brinton, 1959). In addition, flagella are known to be disaggregated by hydrochloric acid (Duncan, 1935, Gillies and Duguid, 1958) and by the detergents Tween 80 and sodium dodecyl sulphate (Kerridge et al, 1962). Fimbriae, on the other hand, are resistant to hydrochloric acid, are synthesised in 0.2% (v/v) Tween 80 broths (Contento and Old, unpublished results) and are morphologically unaffected by sodium dodecyl sulphate. (However, slight differences in the structure of the fimbrial protein not observed by electron microscopy, might have been detected by changes in their antigenicity. This was not investigated). Another difference between the two proteins is that chloramphenicol inhibits the synthesis of flagella (Stocker, 1956) but not of fimbriae (Brinton and La Rosa, personal communication). This latter difference, however, although it may indicate a unique mechanism for the synthesis of fimbriae, can equally well indicate that the sites of synthesis of

fimbrial protein are shielded and inaccessible to chloramphenicol. Finally, it is known from the work of Thornley and Horne (1962) that fimbriae of Proteus are considerably more tolerant to autolytic enzymes than are flagella, the former being morphologically unchanged after three months at room temperature. Since the detailed structure has not been established for the proteins from either fimbriae or flagella, these differences cannot yet be equated with differences in the fine structure of the two proteins.

The fractionation of E.coli fimbriae on DEAE-cellulose was shown to yield two peaks, both haemagglutinating both giving maximum absorption at 280mμ, but only the major one being homogeneous as evidence by agar gel diffusion (it was on this that chemical analyses were performed). The minor peak was consistently eluted at a definite molarity of the eluting buffer, and was obviously not an artefact of non-specific haemagglutination. This minor peak gave two lines by agar gel diffusion tests, one which coincided with the main fimbrial band, the other, occurring some distance from the antigen reservoir; the nature of the antigen causing the second line was not detected because of limited material available. A similar phenomenon of divalent precipitation in a pure preparation of flagella was shown by Gard et al (1955). Fluctuation in the temperature can result in the detection of a number of bands in excess of the number of antigen-antibody systems present (Crowle, 1960). This was not the cause here because no other system was similarly affected on the same plate. It could be explained by

assuming that the minor peak represents fimbriae of similar antigen specificity contaminated by irreversibly-bound O-somatic material (e.g. that portion of fimbrial material extruding through the cell wall) sufficient to alter the surface properties and cause this different behaviour on column chromatography. It might also represent simply a size difference in the fimbriae. It is also possible that this second haemagglutinating peak indicates the presence of two species of fimbriae on the one bacterium. In this connection, it was interesting to find that the major (and, possibly, only) peak obtained from fractionation of Shigella flexneri fimbriae on DEAE-cellulose was eluted at the same molarity of phosphate buffer as the minor haemagglutinating band of E.coli. Fimbriae from Shigella flexneri also showed a weak cross reaction, corresponding to the main fimbrial band of E.coli, with the antiserum prepared against a crude fimbrial preparation of E.coli 23. These findings might be considered proof of the sharing between E.coli and Sh.flexneri strains of a minor "flexneri-coli" fimbrial antigen (Gillies and Duguid, 1958). However, tempting as this comparison is, it must be made with caution because of the paucity of evidence to substantiate this interpretation. The origin of two fimbrial peaks from E.coli is, therefore, not yet explained, but the presence of two fimbrial species per bacterium would not be contradictory to the literature.

It is interesting to speculate on how fimbriae from different species and genera, distinct as judged by serological tests, will behave on this fractionation

procedure. It is possible that the (presumed) different chemical structures will cause each to be eluted at a specific molarity. Attempts to obtain fimbriae from the non-flagellate transductant, S.typhimurium Sa 635 fim + for this purpose (since Salmonella does not show a serological cross-reaction with E.coli and Shigella) were unsuccessful because of low yields insufficient for chromatography. Likewise it is tempting to theorise that fimbriae from different species might show different iso-electric points when subjected to the purification method of Brinton and Stone, 1961, and enable characterisation of the different fimbrial species. These interesting problems, however, are for the future.

The observation that fimbriae from E.coli and Sh.flexneri adsorb on DEAE-cellulose, an anionic exchanger, but not on CM-cellulose, a cationic exchanger, is in agreement with the quantitative amino acid analyses for E.coli fimbriae which revealed a preponderance of acidic amino acids (Brinton, personal communication). These findings also indicate why the highly hydrophilic surface of fimbriae must be neutralised by the addition of divalent cations (such as Mg++) before the fimbriae can approach the hydrophilic surface of red cells and cause agglutination. It is, presumably, this transition from a hydrophilic to a neutral state which is responsible for the pellicle formation which occurs at the broth-air interface in static broth, causing the fimbriate cells in the pellicle to be excluded

Table 26. Quantitative amino acid analysis of fimbriae from E.coli Bam P+. *

ala	33-34	ser	10	tyr	2
thr	21	phe	8	cys	2
asp	20	iso	4	pro	1
gly	17-18	lys	3	try	0
val	14-15	arg	3	met	0
glu	13-14	his	2	NH_3^+	14-15
leu	11-12				

(Results are expressed per molecular weight of 16,800.)

* Unpublished results of Dr. C.C. Brinton, University of Pittsburgh, Pennsylvania.

from the broth.

Weibull and Hedvall's (1953) conclusion that fimbriae of Proteus were related to the cell wall was based primarily on their resistance to the proteolytic enzymes, trypsin and pepsin, i.e. as judged by electron microscopical appearance. These observations have been confirmed for the fimbriae of E.coli, which are stable to pepsin. However, the inability to degrade fimbriae by enzymatic treatment with amylase and pepsin, followed by sodium dodecyl sulphate, which completely disaggregates the cell wall (Weidel et al, 1963), showed that their basic assumption was wrong and confirmed the finding of a different molecular structure. Brinton (1963, personal communication) reports that the amino acids of the fimbriae of E.coli are of L-configuration, so that their resistance cannot be explained by a substitution of the L-amino acids by the unnatural D-isomers. The range of amino acids is not suggestive of an atypical protein, so that the resistance to proteolytic enzymes is probably due to a complex folding of the polypeptide chains, masking the bonds normally attacked and labile. The absence of tryptophane and the presence of only small amounts of tyrosine and histidine (see table 26) might explain the stability to chymotrypsin and small amounts of arginine and lysine the resistance to trypsin.

Now that purification methods are available, it is to be hoped that the chemical structure of the thick fimbriae will be examined in a large number of genera to reveal any quantitative or qualitative differences, which would account

for their serological specificity. It will be useful if both, or either, of the purification methods - column chromatography and isoelectric precipitation - are applicable to the isolation of fimbriae in the presence of flagella. Once this has been proven, the analysis of haemagglutinating and non-haemagglutinating fimbriae from strains such as S.paratyphi B is a problem which is worth investigating, since it may well lead to an explanation of which groups in the protein are specifically involved in this phenomenon. Another aspect obviously worthy of investigation is the different chemical structures of "thin" and "thick" fimbriae, in Klebsiella aerogenes. At the outset of this study, when nothing was known of their chemical structure, except the indications of a cell wall type of structure, it was thought that the difference between the two types was that the MR, thin, fimbriae might be pure protein, whilst the MS, thick, fimbriae might have an additional protective coating or be a lipoprotein structure. This hypothesis has not been upheld by experimental data. The thin fimbriae of Klebsiella strains are apparently trypsin-sensitive, as judged by the somewhat indirect criterion of loss of haemagglutinating activity, which does not occur with the thick fimbriae (Old, unpublished results). This might suggest that the thin fimbriae have a higher content of aromatic and basic amino acids. The difference in thickness between the two types is probably not of especial significance, when one considers that a range of diameters for flagella is

found, specific for each genus (Weibull,1960).

There are many points to be gathered from the chemical study of fimbriae which provide important supporting evidence for the hypothesis that fimbriae are organs of survival. A structure which is to subserve such a role would certainly benefit if it were stable to a multitude of adverse effects, which it might encounter in natural habitats. Such stability is, of course, inherent in the fimbrial structure. Resistance to low pH (Weibull and Hedvall, 1953, Gillies and Duguid, 1958, Brinton, 1959), thermostability (section III of this thesis, Gillies and Duguid, 1958), resistance to autolysis (Thornley and Horne, 1962), to proteolytic enzymes (section III of this thesis, Weibull and Hedvall, 1953) and to detergents are all factors which support the hypothesis that fimbriae are organs of survival.

SUMMARY

1. It has been possible to transduce the property of fimbriation to a number of permanently non-fimbriate strains of Salmonella typhimurium, using pellicle formation, which occurs on serial subculture in aerobic, static broth, as a selection mechanism. The transduction rate, using lysates prepared on wild-type fimbriate S.typhimurium strains, was calculated as 1 per $5 \times 10^7 - 10^8$ infected cells. It was shown that before successful transduction was effected, the recipient strain was required to be motile. This requirement was not satisfied by the presence of paralysed flagella, and is a reflection of the need for positive aerotactic movements by the recipient strain, which will result in its transport to the broth surface.

2. Among 140 naturally-isolated non-fimbriate strains of S.typhimurium, evidence of non-identical allelism in the fim segment of the chromosome was shown. Reciprocal transduction among the mutants suggested the existence of five transductional groups. The order of the mutations involved in these different groups was not ascertained because of limitations in the selection method and physiological differences inherent in the mutants.

3. Reciprocal transduction among 132 non-fimbriate strains of Salmonella typhimurium classified as FIRN, i.e. fim - inl - rha - , did not reveal recombination either to fim+ or rha+. These strains, although epidemiologically independent, are thought to have identical or overlapping mutations in fim and rha. The hypothesis was presented that these arose

from a common fim - rha - parent, and that present-day FIRN strains represent a world-wide, successfully distributed typhimurium type.

4. There was no evidence of a close linkage between fim and any other markers tested, so that the initial hypothesis of a close genetic linkage between fim and rha, to explain the almost absolute correlation of these characters in S.typhimurium strains, was not valid. With the non-fimbriate, non-flagellate derivatives of S.typhimurium LT-2 by ethyl methane sulphonate treatment, called Dubnau strains, indications of cotransduction of fim and fla were presented, but this phenomenon was not examined in detail.

5. One non-fimbriate strain, S.typhimurium Sa 519, produced a small colony fimbriate revertant. Genetic analysis suggested that this was due to suppressor mutation.

6. Induced reversion to a fimbriate state was achieved with the mutagens ultra-violet light and manganous chloride. Limited tests with ethyl methane sulphonate did not induce fimbriate revertants. Of the seven fim - rha + strains of S.typhimurium, three strains reverted to a fimbriate state - Sa 749, 1436 and 1566b. Six of the 132 FIRN strains reverted to fimbriate state, and were characterised as FIRN derivatives. The value of these findings is discussed. Spontaneous mutation to fimbriation was never detected with any non-fimbriate S.typhimurium mutants.

7. A number of fimbriate, non-haemagglutinating strains of S.paratyphi B was transduced to a haemagglutinating state

with transducing lysates from wild-type fimbriate strains of Salmonella typhimurium. This was judged indicative of a gene Ha controlling fimbrial function, but alternate interpretations were discussed.

8. Attempts to demonstrate the presence of a fim episome controlling phase variation in Salmonella typhimurium were without success. Another theory to explain the fim+ ⇌ fim(+) phase variation was presented, and discussed also in relation to the larger number of fimbriate and non-fimbriate types in Escherichia coli.

9. Competition experiments were devised to investigate the transduction procedure for fimbriate cells on a quantitative basis. Small numbers of fimbriate cells were used to challenge large numbers of non-fimbriate cells, both strains being flagellate, in mixed culture under a range of environmental conditions - static broths incubated (a) aerobically, (b) anaerobically, (c) microaerophilically, artificially aerated broths (d) on a reciprocating shaker at 100 oscillations per min. and (e) on a rotator at 12 rotations per min., and, finally, (f) on agar plates. Buffered and unbuffered media were used. The strains used were naturally isolated fimbriate and non-fimbriate strains of Salmonella typhimurium, and pairs of fimbriate and non-fimbriate strains isolated from a transduction mixture. Under conditions favourable to the formation of a fimbrial pellicle, the fimbriate cells outgrew the non-fimbriate cells and formed as much as 80% of the final population after

48 hr. A small outgrowth by naturally isolated fimbriate strains was sometimes detected, not associated with the formation of a fimbrial pellicle, and due to physiological strain differences. The possible mechanisms of this outgrowth are discussed.

10. Similar competition experiments in which the challenger fimbriate cells were non-motile showed that, if the challenger cell was phenotypically fimbriate, it could outgrow large numbers of non-fimbriate, non-flagellated challenged cells even in the absence of motility. This outgrowth was accompanied by the formation of a fimbrial pellicle in the later stages of growth.

11. Competition experiments also revealed the importance of flagella alone. Small numbers of flagellate, motile cells outgrew large numbers of non-motile cells under aerobic static conditions by their ability to form a flagellar pellicle. The outgrowth, however, was less significant than that associated with the formation of a fimbrial pellicle.

12. From these competition experiments and the parallel growth experiments between S.paratyphi B strains with functional and non-functional fimbriae, the advantage bestowed on a cell by its ability to form a pellicle was seen. It is considered that these experiments provide quantitative experimental data supporting the hypothesis that fimbriae are organs of survival.

13. Fimbriae from Escherichia coli were isolated in a pure form by differential centrifugation and by chromatography

on DEAE-cellulose exchanger at a neutral pH, and elution with a salt gradient. The fimbriae were judged pure by five criteria - electron microscopy, ultraviolet spectrophotometry, chemical analysis, agar gel diffusion and biological activity. The relative values of these criteria are discussed.

14. Chemical analysis revealed that fimbriae were protein structures, unrelated to cell wall, capsule or flagella.

15. The range of amino acids detected on two-dimensional chromatography was not suggestive of an atypical protein. The adsorption of fimbriae to DEAE - but not to CM-cellulose suggests a preponderance of acidic amino acids in their structure. The relevance of this finding is discussed in relation to their agglutinating properties and to their resistance to proteolytic enzymes.

16. Chromatography of fimbriae from Shigella flexneri FIaI showed a behaviour different from E.coli 23. The possible relevance of this to sharing of fimbrial antigens is discussed.

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ACKNOWLEDGEMENTS

I should like to express my thanks to Professor Robert Cruickshank for research facilities made available during this investigation and for the interest he has shown. My thanks are particularly due to Professor James P. Duguid and Dr. John F. Wilkinson fortheir guidance and encouragement and for many valuable discussions during this research.

I also express my appreciation to Professor W. Weidel, Max-Planck Institute for Biology, Tubingen, Germany, who afforded me hospitality of his laboratory in March, 1962, and to Professor B.A.D. Stocker for his continued interest, valuable discussions and supply of strains.

My thanks are also due to Miss Dorothy J. Little and Miss Pat D. Nicholson for their excellent technical assistance at various stages of this work, to Mr. G. Wilson for his guidance with electron microscopical and photographic techniques and Mrs. M.J. Wilcox for her secretarial assistance.

I gratefully acknowledge the receipt of a Research Training Scholarship from the Medical Research Council during the academic year 1960-1961, a Younger Research Worker's Travelling Scholarship from the British Council in 1962 and the Chalmers Research Scholarship from Edinburgh University during successive academic years, 1961-1962, 1962-1963.

APPENDIX

Table A. Transduction of fimbriation to *Salmonella typhimurium* FIRN and FIRN' strains.

Recipient strain.	No. of tests giving transduction to *fim*+/ no. of tests performed with phage prepared on donor strain that was:						No. of tests giving mutation to *fim*+ / no. of tests made without added phage.
	Fimbriate		Non-fimbriate				
	FIRP	*Fim*+*rha*-	Dubnau	Group A	Group B	FIRN	
Sal00,							
573	0/6	.	.	.	.	0/1	0/2
576	6/7	.	2/2	.	.	0/5	0/3
577	64/177	0/12	2/2	0/24	.	0/55	0/51
578	0/6	.	0/5	.	.	0/2	0/5
580	0/6	.	.	.	.	0/1	0/2
SL43	0/4	.	.	.	.	0/3	0/2
SL43S	3/14	.	.	.	.	0/10	0/9
582	11/12	.	.	.	.	0/4	0/5
583	4/7	.	.	.	.	0/4	0/4
6683	5/14	.	0/17	.	.	0/13	0/11
6758	1/1	.	1/1	.	.	0/2	0/2
6759	1/9	.	3/3	.	.	0/9	0/8
6760	3/4	.	1/1	0/8	.	0/5	0/4
6822	6/13	.	1/1	.	.	0/9	0/10
6825	1/2	.	.	.	.	0/2	0/2
7471	5/21	0/6	2/2	0/6	.	0/12	0/8
546	1/3	.	1/1	.	0/3	0/4	0/5
549	2/2	.	1/1	.	.	0/2	0/3
805	1/1	.	4/4	.	.	0/1	0/3
807	2/2	.	1/1	.	.	0/3	0/3
808	1/1	.	3/3	.	.	0/2	0/2
809	18/56	0/6	3/3	0/4	.	0/8	0/4
816	1/2	..	3/3	.	.	0/5	0/3
834	3/25	.	1/2	.	.	0/26	0/26
845	5/13	.	1/1	.	.	0/14	0/12
850	1/1	.	1/1	.	.	0/18	0/2
855	2/2	.	1/1	.	.	0/2	0/2
856	1/3	.	1/1	.	.	0/3	0/2
602	1/4	.	1/1	.	.	0/5	0/5
603	2/5	.	1/1	.	.	0/5	0/4
604	1/2	.	2/2	.	.	0/5	0/3
605	1/1	.	2/2	.	.	0/1	0/3
607	2/2	.	1/1	.	.	0/3	0/3
608	1/1	.	2/2	.	.	0/1	0/2
609	7/12	.	2/2	.	.	0/13	0/8
610	3/3	.	2/2	0/2	.	0/3	0/4
611	1/2	.	2/2	.	.	0/2	0/3
612	2/2	.	1/2	.	.	0/2	0/3
613	1/2	.	1/3	.	.	0/3	0/3
614	2/2	.	2/2	.	.	0/2	0/3
616	3/3	.	2/4	.	.	0/5	0/1
618	2/3	.	1/2	0/2	.	0/3	0/4
620	2/5	.	2/2	.	.	0/5	0/7
621	2/3	.	1/2	.	.	0/2	0/3
622	1/2	.	2/2	.	.	0/2	0/3
623	2/4	.	1/1	.	.	0/5	0/5
625	1/1	.	1/1	.	.	0/2	0/2
626	1/4	.	1/1	.	.	0/9	0/3
628	3/3	. .	1/1	0/1	.	0/4	0/4
629	1/1	.	1/1	.	.	0/2	0/2
630	1/1	.	1/1	0/6	.	0/2	0/1
631	1/2	.	2/2	.	.	0/4	0/3
632	6/16	.	1/1	0/4	.	0/15	0/18
633	4/6	.	1/1	.	.	0/6	0/5
634	2/4	.	1/1	.	.	0/5	0/5
635	1/87	.	0/5	.	.	0/59	0/33
636	1/3	.	3/3	.	.	0/3	0/3
639	2/4	.	1/1	.	.	0/3	0/3
701	1/1	.	1/1	0/6	.	0/2	0/2
702	2/6	.	1/1	0/6	.	0/10	0/5
703	1/1	.	1/1	0/6	.	0/2	0/2
704	4/50	.	0/17	.	.	0/44	0/24
705	5/5	.	2/3	0/4	.	0/13	0/12
706	15/82	.	1/1	0/19	.	0/46	0/65
707	2/7	.	1/1	0/4	.	0/9	0/12
708	2/3	.	1/1	.	.	0/3	0/3
709	2/5	.	.	0/3	.	0/7	0/10
710	1/2	.	1/1	.	.	0/3	0/3
711	1/1	.	1/1	.	.	0/2	0/1
713	1/1	.	2/2	.	.	0/1	0/2
714	1/1	.	3/3	.	.	0/2	0/2

709	2/5	.	.	0/3	.	0/7	0/10
710	1/2	.	1/1	.	.	0/3	0/3
711	1/1	.	1/1	.	.	0/2	0/1
713	1/1	.	2/2	.	.	0/1	0/2
714	1/1	.	3/3	.	.	0/2	0/2
715	2/31	.	2/2	0/4	.	0/1	0/4
716	1/1	.	1/1	.	.	0/2	0/2
717	1/5	.	1/1	.	.	0/8	0/4
718	1/1	.	1/1	.	.	0/2	0/1
719	1/3	.	1/1	.	.	0/3	0/1
720	1/14	.	.	.	.	0/5	0/6
721	2/3	.	1/1	.	.	0/4	0/3
722	1/1	.	1/1	.	.	0/2	0/1
733	1/1	.	1/1	.	.	0/2	0/1
734	1/1	.	1/1	.	.	0/2	0/1
735	3/7	.	2/2	.	.	0/16	0/3
738	1/5	.	2/2	.	.	0/4	0/4
760	3/7	.	1/1	.	.	0/16	0/3
645	2/2	.	1/1	.	.	0/2	0/2
652	2/7	.	3/5	.	.	0/7	0/8
653	2/4	.	1/1	.	.	0/5	0/5
654	2/2	.	2/2	.	.	0/4	0/4
932	8/28	.	1/1	0/4	.	0/23	0/23
563	2/2	.	1/1	.	.	0/2	0/3
575	1/1	.	1/1	.	.	0/2	0/2
581	2/6	.	5/5	.	.	0/5	0/5
582	.	.	1/1	.	.	0/1	0/1
598	1/2	.	1/1	.	.	0/3	0/3
874	3/10	.	1/1	0/4	.	0/25	0/13
1366	4/19	.	4/4	0/2	.	0/3	0/2
1516	3/15	0/6	3/3	0/2	.	0/8	0/2
1285	1/9	.	3/5	.	.	0/8	0/9
1286	1/7	.	2/3	.	.	0/5	0/5
1287	5/8	.	3/8	0/2	.	0/5	0/7
1288	1/2	.	1/4	.	.	0/2	0/4
1289	1/3	.	2/4	.	.	0/3	0/5
1290	1/4	.	2/2	.	.	0/5	0/4
1292	0/9	.	0/5	.	.	0/8	0/9
1293	1/2	.	2/4	.	.	0/2	0/4
1294	1/1	.	2/4	.	.	0/1	0/3
1296	3/3	.	1/2	.	.	0/3	0/4
1297	2/3	.	1/1	.	.	0/2	0/3
1298	1/1	.	1/2	.	.	0/1	0/2
1300	1/9	.	4/6	.	.	0/7	0/9
1301	1/9	.	4/5	.	.	0/8	0/9
1303	2/8	.	2/3	.	.	0/7	0/4
1701	2/4	.	.	.	.	0/2	0/1
1702	5/6	.	.	.	.	0/2	0/1
1703	2/31	0/7	.	.	.	0/8	0/5
1704	2/6	.	.	.	.	0/2	0/1
1714	0/19	0/7	.	.	.	0/4	0/3
1720	0/19	0/8	.	.	.	0/4	0/2
1725	3/8	1/7	.	.	.	0/4	0/2
1729	3/8	1/5	.	.	.	0/4	0/2
1376	1/2	0/4	1/1	.	.	0/6	0/2
1569	3/7	0/4	1/1	.	0/3	0/5	0/4
1576	0/11	0/7	4/4	.	0/4	0/9	0/6
1577	3/7	0/4	1/1	.	.	0/5	0/2
1583	5/6	0/3	1/1	.	.	0/2	0/1
1585	0/16	0/12	1/6	.	0/1	0/26	0/7
1642	1/3	0/3	2/2	.	.	0/4	0/1
1643	0/11	0/7	2/4	.	0/4	0/7	0/5
1644	2/11	0/9	3/4	.	0/1	0/10	0/5
1645	0/6	0/5	0/4	.	0/1	0/6	0/3
6624	2/2	.	1/1	.	.	0/1	0/1
6629	1/2	.	1/1	.	0/3	0/1	0/2
6724	1/2	.	1/1	.	.	0/1	0/1
6785	1/2	.	1/1	.	.	0/1	0/1
6788	1/2	.	1/1	.	.	0/1	0/1
6799	1/2	.	1/1	.	0/3	0/1	0/2
6907	2/2	.	1/1	.	.	0/1	0/1
6908	2/2	.	1/1	.	.	0/1	0/1
6929	1/2	.	1/1	.	.	0/1	0/1
6925	1/2	.	1/2	.	0/3	0/6	0/3

The following strains were used as donors in these experiments:
FIRP - Sa 206,375,377,497,741,742,744,745,746,747,748,848,1302,1304,1404,1451 and 1566a
Fim+rha- - Sa 1179,1180,1541 and 6768.
Dubnau - Sa 18, 1134, and 1137.
Group A - Sa 519,749,750 and 1436.
Group B - Sa 1566b and 6631.
FIRN - Sa 100 (43,438,272,577),549,604,609,611,612,614,622,632,635,652,654,702,704,705,706,707, 709,720,735,807,816,834,1290,1298,1300,1376,1576,1577,1583,1642 and 6799.

Table B. Transduction of rhamnose-utilising ability to *Salmonella typhimurium* FIRN and FIRN' strains.

Recipient strain Sa ref.no.	No, of tests giving transduction to *rha*+ / no. of tests performed with phage prepared on donor strain that was:					No. of tests giving mutation to *rha*+/ no. of tests made without added phage.
	rhamnose-utilising		rhamnose-non-utilising			
	FIRP	Groups A,B.	*Fim*+*rha*-	Dubnau	FIRN	
Sa 100,						
573	1/1	.	.	.	0/1	0/1
576	1/1	.	.	0/1	0/1	0/1
577	1/1	.	.	2/4	0/1	0/1
578	1/1	.	.	0/1	0/1	0/1
580	1/1	.	.	.	0/1	0/1
SL43	0/1	.	.	.	0/1	0/1
SL43S	1/1	.	.	1/3	0/1	0/1
582	1/1	.	.	.	0/1	0/1
583	1/1	.	.	.	0/1	0/1
6683	1/2	0/1	.	1/3	0/2	0/3
6758	1/7	.	.	0/2	0/5	0/7
6759	1/1	.	.	0/1	0/1	0/1
6760	.	1/1	.	0/1	0/1	0/1
6822	1/1	.	.	1/1	0/1	0/1
6825	1/2	.	.	.	0/2	0/2
7471	7/7	.	.	0/2	0/4	0/2
528	1/1	.	.	1/3	0/1	0/1
546	1/1	.	.	.	0/1	0/1
549	1/1	.	.	1/3	0/2	0/2
805	1/4	.	.	0/2	0/2	0/3
807	1/6	.	.	0/7	0/6	0/7
808	0/1	.	.	2/6	0/1	0/1
809	1/1	.	.	2/8	0/1	0/1
816	2/9	.	.	0/7	0/7	0/8
834	1/9	0/1	.	0/5	0/9	0/11
845	0/2	1/1	.	1/6	0/3	0/3
850	1/1	1/1	.	0/3	0/2	0/2
855	1/1	.	.	1/5	0/2	0/2
856	0/3	1/1	.	0/4	0/2	0/3
602	2/2	.	.	2/4	0/2	0/2
603	1/1	.	.	2/4	0/1	0/1
604	1/1	.	.	1/4	0/1	0/1
605	1/1	.	.	1/3	0/1	0/1
607	1/1	.	.	0/1	0/2	0/2
608	1/1	.	.	0/2	0/1	0/1
609	1/1	.	.	0/1	0/1	0/1
610	2/2	.	.	0/3	0/2	0/2
611	1/1	.	.	0/2	0/1	0/1
612	1/1	.	.	0/2	0/1	0/2
613	1/1	.	.	0/2	0/1	0/1
614	1/1	.	.	.	0/2	0/2
616	1/4	.	.	1/4	0/5	0/5
618	2/2	.	.	.	0/2	0/2
620	1/1	.	.	0/1	0/1	0/1
621	1/1	.	.	1/5	0/1	0/1
622	1/2	.	.	2/2	0/2	0/2
623	1/1	.	.	2/4	0/1	0/1
625	1/1	.	.	0/2	0/1	0/1
626	1/2	.	.	2/4	0/2	0/2
628	1/1	.	.	0/1	0/1	0/1
629	1/2	.	.	1/3	0/2	0/2
630	1/1	.	.	2/3	0/1	0/1
631	1/2	.	.	2/4	0/2	0/2
632	2/2	.	.	0/2	0/2	0/2
633	1/2	.	.	0/2	0/2	0/2
634	1/1	.	.	1/3	0/1	0/1
635	2/2	.	.	1/4	0/1	0/2
636	1/1	.	.	2/4	0/1	0/1
639	1/4	.	.	0/2	0/5	0/5
701	1/4	.	.	1/2	0/3	0/2
702	1/1	.	.	0/3	0/1	0/1
703	1/1	.	.	0/2	0/1	0/1
704	3/4	1/2	.	0/1	0/6	0/6
705	2/2	.	.	0/2	0/1	0/1
706	1/1	.	.	0/2	0/1	0/1
707	2/2	.	.	0/1	0/1	0/1
708	2/7	.	.	0/4	0/5	0/7
709	1/2	.	.	0/1	0/1	0/1
710	1/1	.	.	0/2	0/1	0/1
711	1/1	.	.	0/2	0/1	0/1
713	1/1	.	.	.	0/1	0/1
714	1/2	.	.	0/2	0/2	0/2
715	1/1	.	.	0/3	0/1	0/1
716	1/7	.	.	0/3	0/4	0/7
717	1/3	.	.	0/8	0/2	0/7

709	1/2	.	.	0/1	0/1	0/1
710	1/1	.	.	0/2	0/1	0/1
711	1/1	.	.	0/2	0/1	0/1
713	1/1	.	.	.	0/1	0/1
714	1/2	.	.	0/2	0/2	0/2
715	1/1	.	.	0/3	0/1	0/1
716	1/7	.	.	0/3	0/4	0/7
717	1/3	.	.	0/8	0/2	0/3
718	1/1	.	.	0/7	0/1	0/1
719	1/1	.	.	0/8	0/1	0/1
720	1/5	.	.	0/6	0/3	0/5
721	1/1	.	.	1/11	0/1	0/1
722	0/3	.	.	1/9	0/1	0/3
733	1/1	.	.	2/10	0/1	0/1
734	1/1	.	.	0/6	0/1	0/1
735	.	1/1	.	2/3	0/1	0/1
738	.	1/1	.	0/2	0/1	0/1
760	.	1/1	.	1/6	0/1	0/1
645	1/1	.	.	0/6	0/2	0/2
652	2/3	.	.	0/3	0/3	0/3
653	1/1	.	.	1/3	0/1	0/1
654	2/10	.	.	0/4	0/8	0/11
932	0/8	0/2	.	0/4	0/7	0/9
563	1/1	.	.	.	0/2	0/2
575	1/1	.	.	.	0/1	0/1
581	1/2	.	.	1/1	0/2	0/2
582	1/1	.	.	.	0/1	0/1
598	0/3	.	.	0/3	0/2	0/3
874	1/1	.	.	0/4	0/1	0/1
1366	2/2	.	.	1/2	0/1	0/1
1516	2/2	.	.	1/2	0/2	.
1285	1/1	.	.	0/4	0/1	0/1
1286	1/1	.	.	0/2	0/1	0/1
1287	0/4	.	.	0/5	0/3	0/4
1288	1/1	.	.	1/2	0/1	0/1
1289	2/2	.	.	.	0/2	0/2
1290	1/3	.	.	2/3	0/2	0/3
1292	4/4	.	.	0/4	0/4	0/2
1293	2/2	.	.	0/2	0/2	0/2
1294	1/2	.	.	0/2	0/2	0/2
1296	0/3	.	.	1/2	0/2	0/3
1297	1/1	.	.	2/2	0/1	0/1
1298	1/3	.	.	1/3	0/2	0/3
1300	1/2	.	.	0/4	0/2	0/2
1301	1/1	.	.	0/2	0/1	0/1
1303	1/2	.	.	.	0/2	0/2
1701	1/1	0/1	.	.	0/1	0/1
1702	1/1	0/1	.	.	0/1	0/1
1703	2/4	0/2	0/1	.	0/1	0/1
1704	1/1	0/1	.	.	0/1	0/1
1714	2/2	2/2	1/1	.	0/1	0/1
1720	2/2	2/2	1/1	.	0/1	.
1725	2/2	2/2	1/1	.	0/1	.
1729	2/2	0/2	0/1	.	0/1	.
1376	0/1	.	.	0/1	0/1	0/1
1569	2/2	.	.	1/1	0/1	0/1
1576	1/2	.	.	1/1	0/1	0/1
1577	2/2	.	.	1/1	0/1	0/1
1583	1/1	.	.	0/1	0/1	0/1
1585	1/2	.	.	0/1	0/1	0/1
1642	1/2	.	.	1/1	0/1	0/1
1643	2/2	.	.	1/1	0/1	0/1
1644	2/2	.	.	1/1	0/1	0/1
1645	1/2	.	.	1/1	0/1	0/1
6624	1/1	.	.	.	0/1	0/1
6629	1/1	.	.	.	0/1	0/1
6724	1/1	.	.	.	0/1	0/1
6785	1/1	.	.	.	0/1	0/1
6788	1/1	.	.	.	0/1	0/1
6799	3/3	.	0/1	.	0/2	0/2
6907	1/1	.	.	.	0/1	0/1
6908	1/1	.	.	.	0/1	0/1
6629	1/1	.	.	.	0/1	0/1
6925	1/2	.	0/2	.	0/1	0/1

The following strains were used as donors in these experiments:

FIRP - Sa 206,375,497,741,1302,1304 and 1451.
Groups A and B - Sa 519 and 1436.
Fim+rha- - Sa 1179 and 1541.
Dubnau - Sa 1134 and 1137.
FIRN - Sa 100 (43S,577),611,614,622,632,654,703,704,705,706,707,709,720,722,734,738,807, 1288,1290,1298 and 1583.